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ERRATA

Vol. 13, page 454, citation 12 should read "Peet-Grady Method. Official method of the National Assn. Insecticide & Disinfectant Mfrs. for evaluating liquid household insecticides. Blue Book [MacNair-Dorland Co., N. Y.] **1939**: 177, 179, 181–183."

Vol. 14, page 375, lines 29 and 30, should read "ethoxide were added 32 g. (0.2 mole) of ethyl malonate followed by 44 g. (0.135 mole) of 6-(2, 4-dichlorophenoxy)hexyl bromide. The free dibasic acid obtained"

Vol. 15, page 72, line 1, "nioculated" should read "inoculated"

Vol. 15, page 89, line 9, "*fruticola*" should read "*fructicola*"

Vol. 15, page 205, TABLE VIII, column 4, "1.5" should read "1.15"

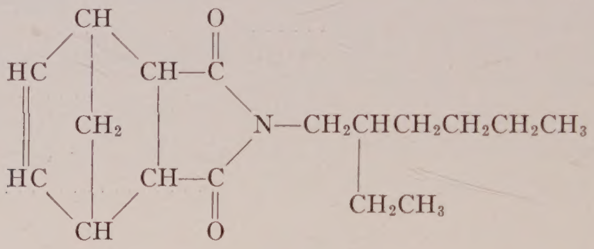
Vol. 15, page 308, line 1 in SUMMARY, "insests" should read "insects"

Vol. 15, page 326, TABLE I, delete last section of body of table on "Southern house mosquito"

Vol. 15, page 334, line 2 in SUMMARY AND CONCLUSIONS, "*Metarrhizum*" should read "*Metarrhizium*"

Vol. 15, page 335, lines 1 and 2, delete "This constituent is capable of causing death to mosquito larvae at less than 200 p.p.m."

Vol. 15, page 337, formula should read



ADDENDA

Vol. 15, page 291, below copyright statement add "This article was preprinted March 5, 1949"

Vol. 15, page 299, below copyright statement add "This article was preprinted March 5, 1949"

EFFECT OF DIFFERENT STORAGE CONDITIONS ON THE GERMINATION OF SEEDS OF CINCHONA LEDGERIANA MOENS.

Lela V. Barton

INTRODUCTION

The keeping quality of *Cinchona* seeds under various conditions is a matter of importance in planting and breeding programs of this genus. It is necessary to keep a viable supply of seeds for continuous nursery propagation to supply seedlings for field plantings of the trees. Also, where extensive hybridization is practiced, seed storage methods, which permit retention of germination capacity of the hybrid seeds until the desirability of the trees produced from a sample planting can be determined, are essential. This might mean a storage period of four to ten years. It has been reported that seeds older than 186 days at the time of planting in Puerto Rico germinated poorly (2). In Uganda, Thomas (7) secured 90 to 98 per cent germination from fresh seeds, 70 to 80 per cent from seeds nine months old, 50 per cent from seeds 20 months old, and only 5 per cent after the seeds had been stored 32 months. Morrison (6) stated that *Cinchona* seeds lost about 5 per cent of their germination power in about two years. Storage was at room temperature in closed containers with calcium chloride or sulphuric acid as a drying agent. No data were given. Airtight containers with calcium chloride also prolonged the viability of *C. Ledgeriana* in Puerto Rico (4). A refrigerator as well as room temperature were used in the last instance but no temperature effect was noted up to 12 months of storage, when 33.5 and 30 per cent germination was obtained.

In an article on *Cinchona* seed storage Kreyer (5) recorded information received from the Biological-Agronomical Institute in Tanganyika that the germination capacity of freshly harvested *Cinchona* seeds reached 99 per cent. However, five months after gathering the viability was reduced by 4 per cent and after 11 to 14 months only about 50 per cent of the seeds were capable of germination. Kreyer stored seeds of *C. Ledgeriana* for 418 days with various drying agents in hermetically closed jars at a temperature of 25° C. Seeds showed high germination capacity when kept in a jar with large doses of sodium bromide, chloride of lime, and a special desiccant called "Adorosu" and with an average dose of caustic lime. None of the controls germinated after 418 days.

Cowgill (3) has done extensive work on *Cinchona*, part of which dealt

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with seed storage. His work will be given in greater detail under "Results and Discussion." In his unpublished article he cited the work of Kerbosch, who stored *Cinchona* seeds under nine vapor tensions. All *C. Ledgeriana* seeds stored at relative humidities of 30 to 60 per cent germinated more than 90 per cent after three years. Higher or lower humidities were less favorable.

The present tests were designed to determine the influence of temperature and moisture on viability. Also two different types of sealed containers were tested.

MATERIAL AND METHODS

The seeds for storage tests were those of *Cinchona Ledgeriana* Moens. obtained from the Guatemala plantation of Experimental Plantations, Inc. The seeds were collected in February 1943, by shaking the maturing inflorescences into bags. The seeds that fell out of the opening capsules were mixed as the different branches were shaken into the same bag. Five trees were used to secure about one-half pound of seed. The capsules were removed but the seeds were not further dried, either artificially or in the sun, before shipping by air mail to Yonkers, N. Y. They were received in Yonkers 20 days after they were harvested in Guatemala.

A moisture determination made by use of a vacuum oven upon receipt revealed 6.0 per cent moisture[1] present in the seeds. In order to adjust the moisture content for determination of the keeping quality when the seeds contained different amounts of moisture, some of the seeds were stored in a desiccator in an atmosphere of 76 per cent relative humidity for 24 hours. At the end of this time the moisture had reached 9.4 per cent. Another seed lot was placed in a desiccator over calcium oxide for 48 hours after which the moisture content was reduced to 4.8 per cent.

The three lots of seeds, containing 4.8, 6.0, and 9.4 per cent moisture, were further divided and samples were stored in the laboratory, in a room held at 5° C., and in a cold room, with an average of approximately $-4°$ C. In the last instance the temperature of the room was usually held within a fluctuation of two or three degrees but on rare occasions, because of the failure of electric power, temperatures above freezing were sustained for short periods. In the laboratory, both light and dark storage were used. Seeds in light storage were in ordinary diffuse light most of each day with some sun early in the mornings. They were in glass bottles or tubes. Dark storage was in a cupboard in the laboratory. Air-dry seeds, i.e., those with 6.0 per cent moisture, were stored in open and sealed containers at all temperatures. Seeds with decreased (4.8 per cent) or increased (9.4 per cent) moisture were stored in sealed containers only.

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[1] The moisture percentages given throughout this paper were calculated on the basis of the dry weight of the seeds.

Two types of sealed containers were used. In one type, glass bottles served for the storage in light in the laboratory and tin cans for dark and 5° C. and $-4°$ C. storage. A large lot of seeds was placed in each container, the cover of which was sealed with sealing wax. When germination tests were made, the containers were opened, samples removed, and then the containers were re-sealed. These seeds were thus subjected to moisture fluctuations as well as atmospheric changes at six-month intervals throughout the storage period. Other seeds, in sufficient quantity for one germination test only, were hermetically sealed in small glass tubes. These were never opened from the time of storage to the time of the germination test. Previous work (1), which will be described more in detail below, has shown a difference in the keeping quality of seeds sealed with and without periodic opening of the containers.

The original seed lot as it was received from Guatemala was composed of a mixture of brown, mature-looking seeds, and very light-colored, almost white seeds. Out of one sample lot separated and counted, 699 seeds were brown and 400 white.

Both brown and white seeds contained embryos, and there were no apparent differences in these embryos as seen under a microscope. However, it was thought advisable to make tests to see whether brown and white seeds had the same germination capacity. Eleven lots of 100 seeds each of each color were planted on top of soil using one lot of 100 seeds for a four-inch pot. In the bottom of each pot was an inch layer of gravel to ensure good drainage. The soil was composed of granulated peat moss, sod soil, and sand in equal parts. A small mount of finely-sifted soil was scattered over the top of the seeds and was tamped down. The seeds were protected by an inverted Petri dish half. The pots were watered from below and were then placed in the greenhouse in a moist bed of granulated peat moss. This ensured a constant water supply for germination without disturbance to the seeds. Shade was provided by covering the pots with brown wrapping paper each sunny day from 8:00 a.m. to 4:30 p.m. After the seedlings had become established the Petri dish covers and the shade were removed.

It was found that 11 pots of brown seeds averaged 70 per cent germination while the average for the same number of white seeds was 26 per cent. There was considerable variation in the germination of the individual lots, i.e., from 52 to 86 per cent for brown seeds and from 16 to 36 per cent for white seeds. These results showed a definite difference in germination capacity of the two colors of seeds. The mixture of colors, as received, were used for storage tests, since it did not seem practical to separate them in such large numbers. This doubtless accounts for some of the variation which will be noted in the data to be presented below. After storage for a time, especially in the laboratory and more markedly for light than for dark storage, the white seeds became brown and indistinguishable in appearance from the more mature lot.

The planting procedure described above was used for routine viability tests made at intervals of six months after storage. The sphagnum medium used by the United States Department of Agriculture in production of *Cinchona* seedlings as well as vermiculite, expanded mica, were satisfactory but showed no advantages over the soil mixture.

To test the germination response of *C. Ledgeriana* seeds to different temperatures, samples were placed on moist filter paper and placed in controlled temperature chambers. Constant temperatures of 15°, 20°, 25°, and 30° C. as well as daily alternations of 10° to 30°, 15° to 30°, and 20° to 30° C. were used. It was shown that these seeds tolerate a rather wide temperature range. There was only 10 per cent germination at 30° C. and one per cent at 15° C., but all other temperatures tried were about equally good. Germinations throughout these tests were somewhat less than on top of the soil mixture in the greenhouse.

Data are now available for seedling production in soil in the greenhouse after storage of the seeds for 3, 6, 12, 18, 24, 30, 37, 42, and 48 months under the moisture and temperature conditions described above.

RESULTS AND DISCUSSION

GENERAL

Table I contains a summary of the per cent seedling production from seeds stored under various conditions up to four years. At the beginning of storage, seeds with 4.8, 6.0, and 9.4 per cent moisture germinated 75, 74, and 66 per cent respectively.

Deterioration in the laboratory was rapid regardless of moisture content of the seeds and in both open and sealed containers. There was no advantage of sealed over open storage. On the contrary sealed storage may have been detrimental, especially when the seeds contained as much as 9.4 per cent moisture.

No difference in keeping quality was noted in this experiment between seeds with moisture contents of 4.8 and 6.0 per cent kept in sealed bottles in light and in sealed tin cans in dark in the laboratory. However, with a moisture content as high as 9.4 per cent, deterioration was more rapid in light than in dark. After 24 months of storage only six per cent of the seeds in light germinated as compared with 42 per cent of those in dark (Table I). The glass bottles or tin cans were opened every time a germination sample was removed. Hence the moisture content tended to be reduced as the storage period lengthened. Also the seeds in dark storage were exposed to daylight for a few minutes at each testing period. The results here are in agreement with those of Cowgill (3), who has reported that daylight and 100 per cent relative humidity at room temperature signifi-

TABLE I

CINCHONA LEDGERIANA. PRODUCTION OF SEEDLINGS IN THE GREENHOUSE FROM STORED SEEDS. FIGURES REPRESENT AVERAGES OF TWO POTS OF 100 SEEDS EACH

Storage				Per cent seedling production after storage for months								
Container	Per cent moisture*	Temperature	Open or sealed	3	6	12	18	24	30	37	42	48
Bottle or tin can	4.8	Lab. (light)	S	66	65	63	55	27	10	2	2	1
		Lab. (dark)	S	63	63	64	46	26	15	6	5	1
		5° C.	S	67	72	62	58	69	76	57	62	62
		−4° C.**	S	67	74	60	71	71	65	50	69	68
	6.0 (Air-dry)	Lab. (light)	O	59	58	57	43†	44	23	17	6	1
			S	66	68	68	50	36	24	13	8	3
		Lab. (dark)	O	70	57	59	53	48	11	15	11	7
			S	59	68	65	49	37	13	5	3	1
		5° C.	O	45	37	18	3	0	—	—	—	—
			S	66	62	68	70	67	62	59	51	70
		−4° C.**	O	68	69	62	53	67	62	41	56	43
			S	58	74	67	59	59	68	54	59	55
	9.4	Lab. (light)	S	58	51	45	19	6	1	1	—	0
		Lab. (dark)	S	53	53	51	50	42	34	19	19	10
		5° C.	S	67	69	60	64	61	74	59	56	60
		−4° C.**	S	63	69	64	56	67	67	50	63	67
Glass tubes	4.8	Lab. (dark)	S	55	60	70	56	50	33	28	21	16
		5° C.	S	57	63	64	45	58	75	37	54	50
		−4° C.**	S	54	72	65	66	68	66	44	53	67†
	6.0	Lab. (light)	S	51	68	62	53	58	53	46	63	65
		Lab. (dark)	S	58	65	64	56	47	37	11	31	12
		5° C.	S	65	67	65	66	54	63	50	64	64
		−4° C.**	S	57	78	63	61	61	71	47	78	64
	9.4	Lab. (dark)	S	45	56	57	18	4	1	0	0	—
		5° C.	S	62	71	71	69	64	71	64	66	63
		−4° C.**	S	63	72	63	54	65	65	54	70	75

* Calculated on basis of the dry weight of the seeds.
** Approximate average temperature.
† Single pot of 100 seeds.

cantly hastened deterioration over corresponding dark storage. These differences were evident after 18 weeks of storage. His failure to obtain differences when storage in 66 per cent relative humidity in the dark was compared with 75 per cent in the light may have been due to the relatively short total storage period of 54 weeks.

A possibility of a reverse effect of light is to be noted for seeds sealed in glass tubes (never opened from the beginning of storage to the time of testing and hence not subject to moisture fluctuation) with 6.0 per cent moisture in light and dark (Table I). An apparent increased reduction in germination capacity has resulted from storage for as long as 37 months in dark (11 per cent germination) as compared with that in light (46 per cent). Subsequent records for 42 and 48 months seem to justify this conclusion. The explanation for this behavior is not immediately apparent. Certainly more work is needed to determine the role of light in the deterioration of *Cinchona* seeds.

Except for the light effect described in the preceding paragraph, the seed moisture content was without effect on keeping quality within the limits of this experiment, i.e., seeds with 4.8, 6.0, and 9.4 per cent moisture gave the same germination response after storage in the laboratory for periods up to four years. A similar statement can be made in regard to the two low temperatures used for storage. This necessarily refers to sealed storage since seeds in open containers are exposed to all the changes in atmospheric humidity from day to day.

In spite of the absence of moisture-content effect, low temperature storage has proved definitely superior to the laboratory for maintaining the germinability of *Cinchona* seeds (Table I). Statistical analysis of the data has shown that up to 48 months of storage, there has been no loss in the germinating capacity of seeds stored sealed at 5° C. or open and sealed at approximately $-4°$ C.

Thus it appears that when the moisture content of *Cinchona* seeds is not above 9.4, temperature is a much more important factor than moisture content in maintaining viability. However, the practice of increasing the life span of these seeds by adding a desiccant to the storage container as described in the literature review in the "Introduction," is certainly a valid one, for the regions where *Cinchona* seeds are produced and stored are apt to be very humid.

That low temperature alone is not sufficient to keep the seeds alive under these conditions is shown by the response to open storage at 5° C. here at the Institute (Table I). The 5° C. room used had a relative humidity of more than 90 per cent during the storage period. Deterioration under these conditions was even more rapid than in the laboratory. There may have been some decline in germination after three months in open containers in the humid 5° C. room. After six months, 37 per cent of the seeds, representing about one-half of the original germination capacity, produced seedlings. After a year, germination was further reduced to 18 per cent and only three per cent of the seeds survived 18 months of such storage. The reason for the sharp decline in viability was to be found in the large amount of moisture absorbed by the seeds. Moisture determinations made after two years' storage revealed 22 per cent moisture. Thus it should not be assumed that any cool place would serve for satisfactory storage of *Cinchona* seeds. A certain degree of dryness is also essential.

Below-freezing storage proved good regardless of whether the container was open or sealed. This method may be feasible where refrigeration is available. It has been demonstrated repeatedly for other seeds here at the Institute that below-freezing temperatures are desirable for seed storage and *Cinchona* is no exception.

ANALYSIS OF VARIANCE

To compare the effects of tin cans and glass tubes, of the three temperatures used, and the length of the storage period upon seed longevity, some of the data were subjected to statistical analysis. The data used are shown in Table II, and the analysis of variance in Table III. In these computations each moisture content was considered as an entity, since differences in behavior of seeds with different moisture contents were obvious (Table I).

TABLE II

Sum of Percentages Obtained from Duplicate Germination Samples of Cinchona Ledgeriana Seeds after Storage

Storage		In tin cans for months:						In glass tubes for months:					
Per cent moisture (dry wt. basis)	Temp.	3	6	12	24	37	48	3	6	12	24	37	48
4.8	Lab. (dark)	125	125	127	51	11	1	110	119	139	100	55	31
	5° C.	134	144	123	138	114	124	114	126	127	115	73	99
	−4° C.*	134	148	119	141	100	136	108	144	129	136	88	98
6.0	Lab. (dark)	117	135	129	73	10	2	115	130	128	94	22	24
	5° C.	132	123	135	134	118	139	130	134	129	108	100	127
	−4° C.*	115	147	133	117	107	110	113	155	126	121	93	128
		In tin cans for months:						In glass tubes for months:					
		3	6	12	18	24	30	3	6	12	18	24	30
9.4	Lab. (dark)	106	106	101	99	83	68	89	111	113	36	8	2
	5° C.	133	138	119	128	122	147	124	142	141	137	127	141
	−4° C.*	125	137	128	111	133	134	125	143	123	108	130	129

* Approximate average temperature.

Seeds containing 4.8 per cent moisture. When seeds containing 4.8 per cent moisture are considered it is found that there are significant differences in their germination capacity at different temperatures and storage periods (Table III). Also there is a significant interaction of these two factors. Examination of the data in Table II reveals that these responses are due to the inferior storage temperature of the laboratory and the consequent decline in viability of the seeds with increased time at this temperature.

Seeds retained their viability equally well whether sealed in tin cans or glass tubes at 5° C. and −4° C. In the laboratory in the dark, however, seeds in tin cans were inferior to those in glass tubes after 24, 37, and 48 months of storage. This accounts for the significant interaction between containers and temperatures. The shortened life span in tin cans as compared with glass tubes is likely due to the gradual increase in moisture content of the seeds in the tin cans. Every time the can was opened to remove a sample for germination tests the dry seeds acquired some moisture from the air. Moisture determination made on this lot of seeds after four years of storage revealed a content of 5.9 per cent. Previous tests have shown the significance of these moisture changes for other seeds, such as dandelion, aster, and verbena (1). In glass vials the original moisture was maintained throughout the storage period since they were not opened until the germination test was made.

TABLE III

GERMINATION OF SEEDS OF CINCHONA LEDGERIANA AFTER STORAGE UNDER VARIOUS CONDITIONS. ANALYSIS OF VARIANCE

Factor	D.F.	Mean squares according to per cent moisture in seeds†		
		4.8	6.0	9.4
Containers	1	98.0	0	496.1
Temperatures	2	2990.5**	3604.4**	5753.3**
Storage periods	5	1865.1**	1771.7**	414.5
Containers × temperatures	2	654.2*	105.6	570.1*
Containers × storage periods	5	120.4	26.9	171.6
Temperatures × storage periods	10	566.1**	802.1**	388.0
Containers × temperatures × storage periods (error)	10	89.7	41.9	131.4
Duplicate pots	36	45.0	31.5	33.3
Total	71			

† Asterisks show variances which give significant F values at the 5 per cent level (*) and at the 1 per cent level(**).

Seeds containing 6.0 per cent moisture. When seeds are stored originally with the moisture content at 6.0 per cent, great differences are manifest between temperatures and storage periods as affecting seed viability (Table III). Also the interaction of these two factors is of a high order of significance. On the other hand, the difference between the tin can and glass tube at laboratory temperature is not so great for these seeds as for those with 4.8 per cent moisture. This indicates that in spite of repeated openings of the tin cans, the moisture content of the seeds had not increased enough to cause significantly more decline in viability than that produced by the initial 6.0 per cent moisture. Since 6.0 per cent represented air-dry seeds at the beginning of the test, substantial moisture changes were not expected and were not found. Actual determination showed a moisture content of 6.1 per cent at the end of the 48-month storage period.

Seeds containing 9.4 per cent moisture. When the initial moisture content of *Cinchona* seeds was as high as 9.4 per cent, temperature played a major role in deterioration under the conditions of the present test (Tables I, II, and III). A secondary factor was found in the type of storage container. It will be seen from the germination percentages given in Table I that there was a definite decline in viability in glass tubes after storage for 18 and 24 months in the laboratory in the dark as compared with tin-can storage. This difference persisted in later viability tests, and is to be seen in the significant interaction of containers and temperatures in the data analysis (Table III). In this case, contrary to container effects on seeds of low moisture content, seeds sealed in tin cans were at an advantage over those in glass tubes, since repeated openings tended to dry the seeds. After four years of tin-can storage at room temperature the moisture in the seeds was reduced from 9.4 to 6.3 per cent.

Cowgill (3) has studied storage temperature and moisture effects on seeds of *Cinchona Ledgeriana*. Samples were germinated at six progressive nine-weeks sampling dates making a total of 54 weeks of storage. The seeds were stored in relative humidity chambers regulated by sulphuric acid solutions to 0, 33, 66, and 100 per cent and at temperatures of 2°, 7°, 16°, ±24° (laboratory), and 32° C. At 0 per cent relative humidity and 32° C., Cowgill found complete loss of viability by the end of 27 weeks, but at the same relative humidity, seeds were capable of germinating 91 per cent after 36 weeks at 2° C., and 71.0 and 82.5 per cent after 54 weeks at 16° and ±24° C. This effect at 32° C. is difficult to understand since 33 per cent relative humidity and 32° C. still permitted 78.5 per cent germination after 54 weeks and 0 per cent relative humidity was not very injurious at 16° and ±24° C.

Cowgill's data showed germination reduced to 3.0 and 2.0 per cent after storage at 100 per cent relative humidity for nine weeks at 2° and 32° C. Seeds were still viable after 54 weeks at 100 per cent relative humidity at 16° and ±24° C., showing 30.0 and 64.0 per cent respectively at that time. The superiority of ±24° over 16° C. for this high humidity storage is not in accord with the results described above, where lower temperatures were advantageous. Also other data of Cowgill's demonstrate that at 33 and 66 per cent relative humidity 7°, 16°, and ±24° C. in that order were best for keeping the seeds viable.

SUMMARY

Seeds of *Cinchona Ledgeriana* Moens. were stored to determine the effects of temperature, moisture content, light and gaseous exchange upon their keeping quality. Data are presented for periods up to and including

four years of storage. Seeds with 4.8, 6.0, and 9.4 per cent moisture were stored in open and sealed containers in the laboratory, at 5° C., and at approximately −4° C.

Deterioration in the laboratory was evident after 18 months and germination was reduced to approximately one-half or less of the original capacity after 24 months. There was no advantage of sealed over open storage at this temperature. When the moisture content of the seeds was as high as 9.4 per cent, there was some evidence of harmful effects of sealing and of light in the laboratory.

Loss of germination capacity also proceeded rapidly in open storage in a humid 5° C. room. On the other hand, seeds stored sealed at 5° C., or open or sealed at approximately −4° C. kept perfectly up to 48 months of storage. Within the limits of this experiment, temperature proved a more important factor than moisture content in the effects on *Cinchona* seeds.

Analyses of variance of some of the data are presented to show the comparative effects of two types of sealed storage containers. Seeds with 4.8 per cent moisture in sealed tin cans which were opened periodically to remove germination samples lost viability more quickly in the laboratory than those in glass tubes which were never opened until the germination test was made. This was thought to be the result of increased moisture content due to exposure to air when the container was opened. Seeds with an initial 6.0 per cent moisture (air-dry) did not change upon exposure to air and consequently showed no significant decline in viability in tin cans over that in glass tubes. Seeds with an initial 9.4 per cent moisture dried out upon repeated short-time exposures to the atmosphere and hence kept better in tin cans than in sealed tubes where the original high moisture was present for the entire storage period.

C. Ledgeriana seeds can be kept successfully for at least four years by maintaining a moisture content of below 9 per cent of the dry weight of the seeds and placing at a temperature between 5° C. and −4 ° C.

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HEXAETHYL TETRAPHOSPHATE AND TETRAETHYL PYROPHOSPHATE: I. THEIR EFFECTS ON PLANTS. II. THEIR TOXICITIES TO INSECTS AND MITES

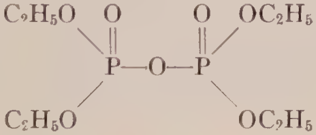
P. W. Zimmerman and Albert Hartzell

Polyphosphates have recently come into prominence as insecticides. So-called hexaethyl tetraphosphate (HETP), originating in Germany, was introduced into the United States during World War II (4). According to recent reports, the commercial preparations known as HETP are probably mixtures of two or more phosphates. Hall and Jacobson (2) claim that the so-called HETP with empirical composition, $(C_2H_5)_6P_4O_{13}$, is composed essentially of tetraethyl pyrophosphate (TEPP) and ethyl metaphosphate with pentaethyl triphosphate as a possible third constituent. They isolated TEPP from the so-called HETP and found the former three to five times as active as the latter. If these findings be true, it appears that all of the insecticidal activity of HETP is due to TEPP.

The following information concerning the formula and probable structure of TEPP is given by Monsanto Chemical Co.:

Formula: $C_8H_{20}O_7P_2$

Probable structure:



Hexamethyl tetraphosphate (HMTP) was also tested and found not as toxic to plants as HETP but had good insecticidal value.

As is usual with a new insecticide, growers of greenhouse plants did not fully agree on the phytotoxicity of these phosphates. There were claims that the sprays caused abnormal rose buds and that leaves were injured. The "bull head" type of buds described was thought to be similar to those which grow when traces of physiologically active gases like ethylene are present. Since HETP is thought to release ethylene gas when broken down by heat, it was assumed that the injury to roses might have been due to this gas. The experiments reported herewith were designed to test the phosphates and their decomposition products for physiological activity when applied to plants, and for insecticidal value.

The phosphate compounds used in the course of these experiments

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were manufactured principally by Monsanto Chemical Co., St. Louis, Mo., and Victor Chemical Works, Chicago, Ill. In some cases they were sent to the Institute directly from the manufacturing company, but a number of chemicals were sent by Dr. W. E. Blauvelt of Cornell University, Ithaca, N. Y. Monsanto Chemical Co. sent TEPP, and Dr. Blauvelt sent HETP, which he stated came directly from Monsanto Chemical Co. Victor Chemical works supplied the Institute directly with HETP. Dr. Blauvelt sent a commercial preparation called Vapotone which was sent to him by California Spray-Chemical Corp., Elizabeth, N. J. Vapotone was labeled "50% hexaethyl tetraphosphate."

I. EFFECTS ON PLANTS

The first experiments involved the effects of HETP when applied directly to plants and when vaporized by heat. Tomato plants used as test objects were placed in glass cases or bell jars where the chemicals were warmed or heated sufficiently to cause vapors to arise.

The first evident effect on tomato plants was injury on leaf blades, having the appearance of cooked tissue. This is the kind of injury which can be caused with concentrated chemical vapors though not specific for any one compound.

The second effect was epinasty of tomato leaves. This was most evident on leaves of a plant placed under the bell jar four hours after the chemical was heated. In the latter case there was no injury on the leaves but epinasty was pronounced as shown in Figure 1. It was assumed that after four hours the concentration of the compound causing injury had been reduced through absorption by the plant or adsorption on the glass so that it was no longer toxic though a physiologically active gas remained. The response of the plant resembled that which can be induced with ethylene gas. Since it has been reported that HETP when subjected to a temperature of 135° C. or more decomposes with the evolution of ethylene, it may well be assumed that the epinastic response was due to this gas.

HMTP applied under bell jars with heat did not cause injury to leaves or epinasty as described for HETP and TEPP. Upon decomposition with heat HMTP did not liberate a physiologically active gas as did HETP. These results lend support to the assumption that under certain conditions HETP releases ethylene gas.

Similar experiments to those described with bell jars were performed where large glass cases (4 ft. × 3 ft. × 3 ft.) were used. Plants were placed in the cases with the chemicals on metal plates hot enough to cause evolution of vapors. The vapors evolved by this method caused slight injury to tomato leaves followed by epinasty on the same plants. Leaves of roses in the same case were injured and the young petioles curled downward. The old leaves of roses turned yellow and dropped from the plant. Both responses are known to occur when rose plants are exposed to ethylene gas (1).

TEPP was used as described for HETP with similar results. The injury induced was approximately equal from both chemicals but the epinasty with TEPP was less. It must be mentioned, however, that the temperature to which the chemicals were heated was not under control and was probably not the same in both cases.

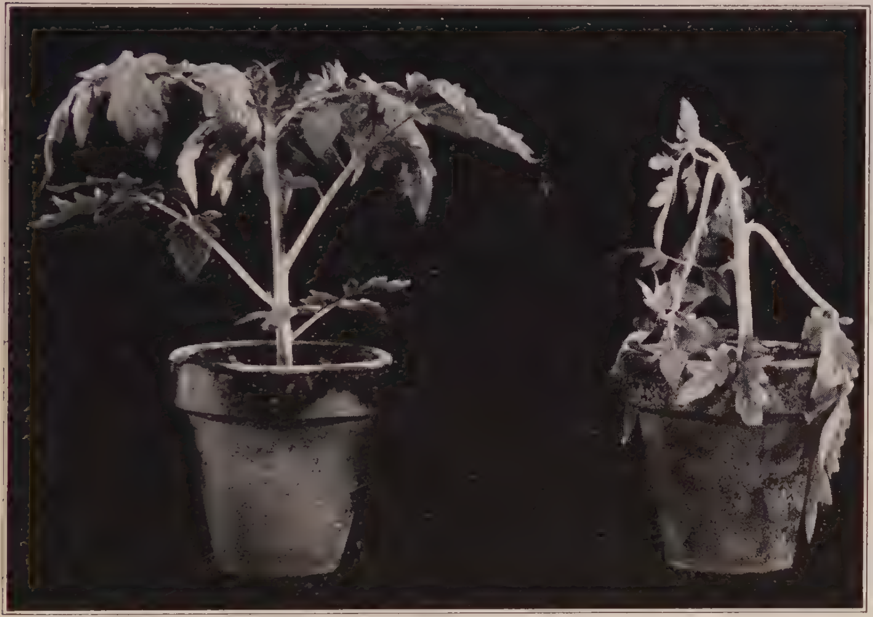

FIGURE 1. Tomato plants to show effects of HETP 24 hours after three drops of the chemical were heated under bell jar. Control on left. The plant on right was placed under the bell jar four hours after the chemical was heated by placing three drops in a watch glass on an inverted hot crucible. The response resembles that which could be induced with a trace of ethylene gas.

The injury on the tomato leaves described above was similar to the injury caused by spraying the plants with high concentrations of the chemical or applying water solutions to the soil. The chemical enters the plant through the roots, passes up the stem, and then into the leaves where it kills the tissue. The soil of four-inch pots was treated with 50 ml. of solution, containing 25, 50, 100, 250, and 500 mg. each of the chemicals. The tomato plants growing in the soil were approximately eight inches in height.

Within six hours after the solutions were applied to the soil, all tomato plants that were not badly injured showed epinasty of the leaves. This response was evident for 24 hours and then the plants began to recover. Within 48 hours no epinasty could be observed.

All treatments with TEPP except 25 mg. per pot killed the plants, and this treatment caused slight injury at the margin of the leaf blades.

The plants treated with HETP were killed only with 250 mg. and 500 mg. per pot.

The sample called Vapotone was applied at twice the concentration given for HETP because it was labeled 50 per cent HETP. With this dosage the results were like those given for HETP.

HETP and TEPP were dispensed in a greenhouse with a large number of species of plants by means of a stationary "Fumeral Diffuser" manufactured by Fumeral Co., Racine, Wis. This diffuser with 100 pounds or more of pressure emits small particles of spray solution which float to all parts of the house. The particles are not as fine as those produced by an aerosol dispenser, but they remained in the air for more than an hour.

The solution dispensed was made up of 30 ml. of TEPP and 70 ml. of water. Only 75 ml. of the solution were forced out of the glass container and the volume of the house was 4250 cu. ft. The plants were placed on the greenhouse benches so that some of them were in direct line with the spray being diffused with 200 pounds of pressure from a nitrogen tank. These plants were hit directly by the spray solution while those in other locations were exposed to solution particles floating in the air.

The following species in direct line with the spray were injured: *Amaryllis* sp., *Azalea* sp., *Chrysanthemum* sp., *Fragaria* sp. (strawberry), *Kalanchoe daigremontiana* Hamet et Perrier, *Lycopersicon esculentum* Mill. (tomato), *Oxalis* sp., *Polygonum* sp. (smartweed), *Rosa* sp., *Stellaria media* (L.) Cyrill. (chickweed), *Stevia* sp., *Tigridia* sp., *Trifolium hybridum* L. (Alsike clover), and *Tropaeolum* sp. The species not injured were as follows: *Begonia semperflorens* Link & Otto, *Hibiscus rosa-sinensis* L., *Hymenocallis* sp. (spider lily), and *Passiflora* sp. (passion flower).

Plants injured at distant locations in the house were as follows: *Calonyction* sp. (moonflower), *Clematis* sp., *Ipomoea purpurea* (L.) Roth (morning glory), *Lycopersicon esculentum* Mill., *Mentha* sp. (mint), *Petunia* sp., *Quercus* sp. (oak), and *Saxifraga* sp. The species not injured were as follows: *Camellia* sp., *Citrus* sp. (orange seedling), *Daphne odora* Thunb., *Epidendrum* sp. var. O'Brienianum, *Ilex* sp. (holly), *Narcissus* sp. (daffodil), *Nephrolepsis* sp. (fern), *Saintpaulia ionantha* Wendl. (African violet), *Solanum dulcamara* L. (nightshade), and *Vanilla* sp. (vanilla orchid).

All species injured by the direct spray recovered except *Kalanchoe* and tomato plants. Rose plants lost all leaves, but the stems were not injured and new leaves appeared within a normal period of time. Flowers on the recovered roses appeared normal.

There was no evidence of the presence of physiologically active gases which cause epinasty of leaves. The abnormally high dosage of TEPP was used to test the theory that a spray of TEPP in a greenhouse might break down and liberate ethylene gas.

In another experiment 18 ml. of TEPP were dissolved in 85 ml. of water and dispensed from the Fumeral Diffuser as described above. Practically all species listed above were used in this experiment. There was less injury than with the higher dosage. Only tomato plants were badly injured. Except where the plants were in direct line with the dispenser they recovered within a few days. No leaf epinasty occurred.

HETP was dispensed from the Fumeral Diffuser in the same greenhouse and the results compared with those involving TEPP. HETP is slightly less toxic to plants and there was also a difference in insecticidal activity. Fifteen milliliters in 400 ml. of water were dispensed under 300 pounds' pressure within a period of four minutes. The same species of plants listed above were used in this experiment. Tomato, strawberry, and chrysanthemum species in direct line with the main stream of spray were injured. The tomato plants were most severely injured, but the stems were not killed and the plants eventually produced new shoots. Tomato plants were injured at all locations in the greenhouse. No other species where not in direct line with the spray were injured and there was no evident epinasty of leaves.

TEPP and HETP (from Vapotone) were mixed in a spray solution [10 ml. TEPP, 10 ml. Vapotone (50 per cent HETP), 10 ml. sesame oil, and 400 ml. water] and applied with the Fumeral Diffuser. The same species of plants were used, and the results were similar to those of the other experiment. In the first experiments 75 ml. of solution were used (30 ml. of HETP in 70 ml. of water) while in later experiments 375 ml. were dispensed. The concentration of the chemical solutions differed, but the ultimate effects were similar with four times the dilution with water.

It appears from the results of the experiments reported herewith that when applied as sprays or dispensed as aerosols, HETP and TEPP do not cause hormone-like responses of plants. When heated to a temperature of 135° C. or more or otherwise caused to disintegrate to liberate ethylene gas, injury or undesirable hormone-like effects may result.

The epinastic response induced by HETP and TEPP when applied as a water solution to the soil indicates that ethylene might be liberated through decomposition without the application of heat. It is conceivable that in practice enough solution from a heavy spray might get into the soil to affect plants. This, however, could not be proved from the results of experiments reported herewith.

II. TOXICITIES TO INSECTS AND MITES

Hall and Jacobson (2) isolated TEPP from HETP and found that the insecticidal activity of TEPP was five times that of HETP.

Smith and others (4) have tested aerosols of HETP in methyl chloride in greenhouses for the control of insects and mites. They found that dosages of 1 g. per 1000 cu. ft. were toxic to greenhouse white fly, mealy bug, red spider mite, and several species of aphids, but this concentration was not found to be effective against the broad mite.

Ludvick and Decker (3) have tested a number of organic derivatives of phosphorus acids as sprays against aphids. Eight compounds were found to be superior in toxicity to nicotine alkaloid, among the most promising of these were HETP, TEPP, and *sym.* tetrapropyl phenyl triphosphate. The methyl acid phosphate ester gave only a 51.4 per cent kill of *Myzus persicae* at a dilution of 1 to 500 in water.

PRELIMINARY LABORATORY TESTS ON INSECTS AND MITES

Aqueous solutions of HETP were sprayed on detached bush bean (*Phaseolus vulgaris* L. var. *humilis* Alef.) leaves infested with red spider mite (*Tetranychus bimaculatus* Harvey), and also on detached nasturtium (*Tropaeolum* sp.) leaves infested with *Aphis rumicis* L. The sprayed leaves were placed in Petri dishes with a moistened filter paper disc in each cover. Counts of living and dead aphids and red spider mites were made at the end of 24 hours. The results appear in Tables I and II.

TABLE I

TOXICITY OF HEXAETHYL TETRAPHOSPHATE* TO RED SPIDER MITE ON DETACHED BEAN LEAVES WITHOUT A WETTING AGENT. SOLUTION MECHANICALLY AGITATED BY A WARING MIXER

Parts of water to 1 part HETP	No. mites counted	Per cent dead	Average
1600	124	97	97
	80	97	
6000	65	90	78
	124	71	
Control	80	6	7
	76	7	

* Victor Chemical Works, Chicago, Ill.

The sprays gave satisfactory control of both *Aphis rumicis* and red spider mites (larvae and adults) at dilutions of 1 to 1600 with water, under laboratory conditions.

HMTP at a dilution of 1 to 2000 gave a perfect kill of red spider mite on detached bush bean leaves.

TABLE II

TOXICITY OF HEXAETHYL TETRAPHOSPHATE* TO APHIS RUMICIS ON DETACHED NASTURTIUM LEAVES WITHOUT A WETTING AGENT. SOLUTION MECHANICALLY AGITATED BY A WARING MIXER

Parts of water to 1 part HETP	No. insects counted	Per cent dead	Average
1600	45	95	95
	48	90	
6000	57	87	88
	43	88	
Control	48	6	6
	48	6	

* Victor Chemical Works, Chicago, Ill.

TABLE III

EFFECT OF FUMIGATION OF POLYPHOSPHATES ON RED SPIDER MITE AND MEALY BUG IN GREENHOUSE

Formula	Amt. diffused, ml.	Days after fumigation	Red Spider Mite			Mealy bug			
			Total	% Dead	Host plant	Total	Moribund	% Dead	Host plant
30 ml. TEPP 70 ml. water	75	1	152	93	Bush bean	54	22	22	Moonflower Chrysanthemum
18 ml. TEPP 85 ml. water	75	1	282	98	Bush bean	326	165	49	Moonflower
		4	—	—	—	100	8	54	Chrysanthemum
18 ml. HETP 400 ml. water	375	1	250	98	Morning glory Nightshade Rose	147	16	29*	Moonflower Chrysanthemum
		1	—	—	—	131	14	35**	
15 ml. HETP 10 ml. sesame oil 400 ml. water	375	1	250	100	Nightshade	150	0	12	Moonflower Chrysanthemum
10 ml. Vapotone (50% HETP) 10 ml. TEPP 10 ml. sesame oil 400 ml. water	375	1	258	99	Morning glory	56	3	89†	Moonflower Chrysanthemum

* Insects examined on plants from end of greenhouse farthest removed from diffuser.
** Insects examined on plants near diffuser.
† High mortality due to the fact that the population of mealy bugs consisted mostly of larvae.

EFFECTS OF GREENHOUSE FUMIGATIONS ON INSECTS AND MITES

The results of greenhouse fumigation tests on red spider mite and mealy bug (*Pseudococcus citri* Risso) appear in Table III. All of the mixtures gave satisfactory control of red spider larvae and adults, but did not inhibit the eggs from hatching.

The degree of control obtained with mealy bugs seemed to be dependent on the relative proportion of larvae to adults. When the population consisted mainly of adults, low kills resulted, but when the population was composed mostly of larvae, a comparatively high kill was obtained. The fumigants in some instances caused a large proportion of the adults to become moribund, and although many individuals were capable of moving legs and antennae even after four days, they could not right themselves and doubtless perished. The eggs were resistant to all mixtures tested. The distance of infested plants from the diffuser did not appreciably affect the kill of mealy bugs in the present tests, although this no doubt would be an important factor in a larger greenhouse.

A green aphid (*Aphis gossypii* Glover) feeding on morning glory was effectively controlled (95 per cent kill in 24 hours) with the 30 ml. TEPP and 70 ml. water mixture, while *Macrosiphoniella sanborni* Gill. on chrysanthemum proved more resistant with a kill of only 11 per cent in 24 hours and 95 per cent in 48 hours. Counts of approximately 100 individual insects were made in each of these two aphid tests.

SUMMARY

Hexaethyl tetraphosphate and tetraethyl pyrophosphate were applied to plants as vapors, sprays, and as solutions to the soil. When heated in closed containers the chemica's first injured the leaves and later caused epinastic responses. The latter response is assumed to be associated with a decomposition product, ethylene gas. Hexamethyl tetraphosphate, used in the same way, did not injure the leaves or cause epinasty.

No epinasty of leaves was observed in a greenhouse where water solutions of the chemicals were diffused by means of a Fumeral Diffuser.

When water solutions of TEPP containing more than 25 mg./50 ml. of water were applied to the soil in which tomato plants were growing, the plants were badly injured or killed. Lower dosages caused epinasty of leaves. When applied to soil, water solutions of more than 100 mg. of HETP per 50 ml. of water killed tomato plants. Lower dosages caused epinasty of leaves.

All fumigation formulas of HETP and TEPP (the lowest being at the rate of 18 ml./400 ml. of water solution) used for greenhouse application gave satisfactory control of red spider mite larvae and adults but did not kill the eggs. The kill of mealy bugs was in proportion of larvae to adults,

the latter being the more resistant. Approximately 90 per cent of larvae were killed with the best formula. Eggs of mealy bug were not killed. With repeated dosages, however, it is assumed that mealy bugs could be controlled or the population greatly reduced.

Preliminary tests with HMTP at a dilution of 1 to 2000 in water gave perfect kills of red spider mite.

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ADDITIONAL TESTS OF PLANT PRODUCTS FOR INSECTICIDAL PROPERTIES AND SUMMARY OF RESULTS TO DATE

Albert Hartzell

As the supply of pyrethrum and rotenone in the United States depends largely on importations from the Far East, Africa, and South America, during the war these supplies were markedly reduced and in some instances completely cut off. Consequently serious efforts were made to replace these imported insecticides. Nicotine, an insecticide derived from tobacco, a plant indigenous to the United States, and certain organic synthetics were substituted to some extent, but pyrethrum and rotenone were not adequately replaced where an insecticide non-poisonous to warm-blooded animals was desired. This demand tended to stimulate insecticidal investigation of other plant sources, particularly of those species of plants indigenous to Central and South America.

The present paper is a continuation of work previously reported (24, 25, 54) on plant products possessing toxicity to insects, together with a brief summary of 400 species and varieties of plants tested to date in this survey. Products of a number of species included in this survey have been tested on various species of insects by other investigators, but comparatively few mosquito larvicides have been reported.

HISTORICAL REVIEW

McIndoo (35) has reviewed the literature up to 1941 on plants of possible insecticidal value. No attempt will be made here to cite the voluminous literature on such well known plant insecticides as pyrethrum, rotenone, and nicotine as the literature pertaining to these insecticides is fully reviewed in publications of the Imperial Bureau of Entomology (20).

Toxicity studies were made of 35 species of Chinese plants by Lee and Hansberry (32) using chiefly silkworm (*Bombyx mori* L.), Mexican bean beetle (*Epilachna varivestis* Muls.), and bean aphid (*Aphis rumicis* L.) as test insects. Products of 18 species of plants killed at least 50 per cent or more of the species of insects tested. The Chinese yam bean (*Pachyrrhizus erosus* Urban) seemed to offer the most promise since the seeds can be collected in quantity in Latin America where the tubers are used as food. The seeds of *Millettia pachycarpa* Benth. were found also to be potent as insecticides. The following other species of plants are listed by these authors as justifying further work: *Delphinium delavayi*

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Fr., *Tripterygium forrestii* Loes., *Phytolacca acinosa* Reb., three species of *Aconitum*, and an unidentified species each of *Celastrus* and *Palaquium*.

Ether extracts of the seed of yam bean were found by Meyer (37) to be very toxic to the caterpillars of the cabbage butterfly (*Crocidolomia binotalis*).

Norton (39) isolated seven compounds from yam bean. One was identified as rotenone, but the others have not been fully determined.

A botanical survey conducted in Mexico, Guatemala, and El Salvador shows the existence of six species of yam beans (*Pachyrrhizus*), three of which are new species. The group is extremely variable taxonomically and in insecticidal value according to Clausen and Hansberry (12).

Following this work, Hansberry and Clausen (23) investigated the properties of a number of miscellaneous plants. With the exception of the seeds of *Amorpha fruticosa* L. and *Tephrosia virginiana* (L.) Pers., all plants collected in New York State were non-toxic. The roots of *Tephrosia* spp. from Mexico used as a dust were moderately toxic, killing less than half of the larvae of Mexican bean beetle in four days. *Clibadium surinamense* L. (*C. sylvestre* Baill.) and *Pithecellobium trapezifolium* Benth. (*P. jupanba* Urb.) from Dominica were slightly toxic to silkworms. The first named plant species is a tree, the last named is a vine. *Tephrosia vogelii* Hook. f. leaves contained 1 per cent rotenone and 7.6 per cent other extractives. Dried pennyroyal (*Pycnothymus rigidus* [Bart.] Small) plants were not effective on bean beetle and aphids. The sap of *Hyra polyandra* Baill. obtained from Mexico gave moderate kills of *Aphis rumicis*, but is extremely caustic and irritating to the human skin, eyes, and respiratory passages.

Field tests of the Thunder God Vine (*Tripterygium forrestii* Loes.) were made by Tien-Hsi Cheng (11) against the melon leaf beetle (*Rhaphidopalpa chinensis* Wei). Plants dusted with the powdered bark of root resulted in only two or three per cent of the plants being seriously attacked by the melon beetle, so that the dust has a strong repellent effect. The aphid population also was reduced from 3 to 70 per cent. The dust was effective against the cabbage flea beetle. As this dust is not toxic to domestic animals, it is considered a highly promising insecticide in China.

In 1939 it was found by Allen and others (2, 3, 4, 5, 6) that the seed of sabadilla (*Schoenocaulon* spp.), known as a vermifuge since the sixteenth century, was among the most promising materials tested as insecticides. Kerosene extracts of sabadilla seed were highly toxic to houseflies, the kill and knockdown comparing favorably with pyrethrum. Toxicity is associated with the veratrine group, compounds of several alkaloids known as cevadine, veratrine, veratridine, sabadelline, and sabadine occur in the endosperm, embryo, and seed coat. Veratridine was found to be a highly toxic constituent; cevadine exhibited considerably less toxicity, according to Ikawa *et al.* (28). Cevine and the seed oil were non-toxic, but the latter possessed knockdown power. Both veratridine and cevadine, as well as mixtures of the remaining alkaloids, gave very quick and complete knockdown of houseflies. Cevine and cevine dibenzoate showed no toxicity in kerosene to houseflies.

By increasing the temperature during extraction, the toxicity of sabadilla is increased. Heating the powdered seed to 150 ° C. for one hour or treating with alkali increased the toxicity (30). The toxicity of powdered sabadilla seed also increased during storage. Kerosene extracts of the seed retained their toxicity in brown glass containers for a period of two years, while extracts exposed to sunlight deteriorated rapidly.

In feeding experiments with rats, sabadilla was found to be much less toxic than DDT or rotenone (30). Because of the sternutatory action produced during inhaling, its application may be somewhat limited.

Sabadilla appears to be especially toxic to species of Heteroptera and Homoptera (2, 14, 16, 17, 18, 19, 30, 44, 45, 48, 52). Dusts containing 10 to 20 per cent of sabadilla have been reported as controlling squash bug, green stinkbug, milkweed bug, chinch bug, hairy chinch bug, tarnished plant bug, harlequin bug, and various *Lygus* species. Among Homoptera the potato leafhopper and the grape leafhopper are effectively controlled by sabadilla dusts. Other insects that have been controlled by sadabilla are cattle lice, cranberry worm, cabbage worms, gypsy moth, and the red-legged grasshopper (10, 30).

On the other hand, sabadilla was ineffective in the control of pear psylla (22) and cauliflower worms (27).

Petrischeva (42) determined the insecticidal action of 200 species of plants of the Siberian flora and found 19 species with well marked insecticidal properties. Among these, *Aconitum barbatum* and several species of *Delphinium* showed antiparasitic activity in small doses for insects and no toxicity for warm-blooded animals. *Inula helenium*, *Artemisia vulgaris*, *Nepeta nuda*, *Ziziphora clinophodioides*, and three species of *Veratrum* showed the most pronounced insecticidal properties. The test insects included mosquito larvae, flies, lice, bugs, cockroaches, and ants. The plant extracts were tested for both stomach poison and contact action. Details of the methods of testing and per cent kill obtained on insects are not given by Petrischeva. *Polemonium coeruleum* is reported as a new emulsifier for insecticides.

False indigo or cat willow, *Amorpha fruticosa*, which grows from Pennsylvania to the Rocky Mountains was found by McCrory (33, 34) to have limited insecticidal power. The greatest amount of insecticide is located in the seed coat and acetone was found to be the best solvent. Most samples were not more than 50 per cent as effective on *Aphis rumicis* than was a similar extract from Derris roots containing 5 per cent rotenone.

The toxic principle found in the seed coat of *Amorpha fruticosa* has not been isolated, but color reactions are indicative of the rotenoid group. No rotenone has been identified, however, but a glycoside has been isolated which reacts to the rotenoid tests. The term "amorpha" has been used to designate it by Brett (8, 9). Amorpha dust gave kills of 87 to 97 per cent on chinch bug, cotton aphid, bean leaf beetle, and spotted cucumber beetle. It gave a fair control (kills of 32 to 69 per cent) of Colorado potato beetle, potato leafhopper, tarnished plant bug, squash bug, and blister beetle. It failed to control cabbage looper, grasshopper, chrysanthemum aphid, mealy bug, red spider, chicken mites, and dog fleas. Extracts of amorpha in water were found to be more toxic to mosquito larvae than was one per cent rotenone. Acetone extracts of the fruit of *Amorpha fruticosa* were found to be repellent to chinch bug and striped cucumber beetle but only slightly repellent to two species of mosquitoes, *Aëdes aegypti* and *A. albopictus*.

The powdered root of sweet flag (*Acorus calamus*) was found by Mironov (38) to be toxic to anopheles mosquitoes and houseflies. It was found also effective against bird lice, clothes moth, and bedbug by Subrahmanyam (46).

Material prepared from tropical plants of the genus *Ryania*, particularly *Ryania speciosa* Vahl., was found by R. E. Heal to be toxic to insects. The material is known as Ryanex, and is a product of Merck & Co., Inc. Pepper and Carruth (41) report it effective in the control of the European corn borer, but Hamilton (22) found it ineffective in the control of pear psylla. Ryanex had an effect comparable to DDT in reducing the twig infesting larvae of the oriental fruit moth, according to Wheeler and LaPlante (53), but did not exhibit the residual action of DDT.

Pendse, Phalnikar, and Bhide (40) report that *Spilanthes acmella* was lethal at 10 p.p.m. to anopheles mosquito larvae. Spilanthol has been isolated.

An isobutyl amide of an unsaturated C_{10} acid has been isolated by Acree, Jacobson and Haller (1) from the roots of *Erigeron affinis* DC. The amide is called affinin and has the same order of paralyzing action to houseflies as pyrethrum and is toxic to several other species of insects.

Home stored beans infested with bean weevil were protected against bean weevil attack by the addition of 1 g. of black pepper to 200 g. of beans, in experiments conducted by Lathrop and Keirstead (31).

McIndoo (36) has reviewed the literature on the castor bean plant as a source of insecticides. Haller and McIndoo (21) conclude that an insecticide principle possibly is present in castor bean plants only under certain conditions with respect to variety, cultural practice, and environment. The accepted use of this plant as a source of insecticide awaits the isolation, identification, and methods of analysis of the specific substance toxic to insects. They call attention to one exception, however, that ricinine, an alkaloid present in castor bean seed, is highly toxic to codling moth larvae.

Oil of arachis (peanut) was found to be the most toxic and the best emulsifier in a series of oils tested by Viel (51) on San José scale.

Jones and Plank (29) discovered that the seed of mamey (*Mammea americana* L.) contains a principle highly toxic to certain insects. Toxicity was found to be due to an ester, the alcohol and acid components of which are non-toxic. With *Diaphania hyalinta* L. the toxicity ratio of pyrethrum resin to mamey seed resin is 7.5; with *Diabrotica bivittata* F. the ratio is about 5.

Extracts of certain tropical species of *Euphorbia* have been reported by Vanderplank (50) as toxic to insests.

MATERIALS AND METHODS

The crude botanical drugs were obtained from S. B. Penick & Company, New York, N. Y.

The following species of plants were obtained from Boyce Thompson Arboretum or grown in the Institute gardens and greenhouses or collected locally: *Anchusa*, *Astilbe*, avocado, black acacia, black walnut, camellia, chive, *Cissus*, dusty miller, *Fraxinus*, *Kalanchoe*, oak, potato, rubber plant, wandering Jew, watercress, and willow. Irish moss was obtained from the sea off the coast of Maine. Tung seed and tung oil were obtained from the American Tung Oil Association, Poplarville, Miss.

METHOD OF EXTRACTION

The dried plant products, including whole botanical drugs, were ground into a fine powder in an electric grinder (Hobart food cutter). The powdered plant materials were placed in extraction thimbles, weighed on an analytical balance, and extracted with acetone in a Wiley Soxhlet extractor overnight (20 hours). For the strongest concentration of acetone extract tested, a quantity of extract equivalent to 4 g. of dry material was made up to one liter. After extraction, the acetone was evaporated to give 3 cc. of extract. Using 1.5 cc. of extract to 1000 cc. of water a concentration of 4000 p.p.m. was obtained. The range of concentrations tested, based on dry weight of powder at the start, was 16 to 4000 p.p.m.

BIOLOGICAL TESTS

Tests made with mosquito larvae (*Culex quinquefasciatus* Say) were conducted according to methods previously described (24). The mosquito eggs[1] were shipped from Orlando, Florida, to Yonkers, N. Y., via air mail. When the larvae hatched, they were fed powdered Rockland rat food (300 mg. to 4 l. of water) and blood albumin (500 mg. to 4 l. of water) on alternate days. Larvae from five to six days old were used for testing.

[1] Obtained through the courtesy of Dr. C. H. Bradley.

Test tubes (25 cc. capacity) containing 10 larvae each in the solution to be tested and the controls in tap water alone, and acetone and water were placed in an oven at 29° ± 1° C. overnight (20 hours). All tests were run in duplicate and in at least four different concentrations differing by a dose ratio of 2. Acetone controls consisted of 1.5 cc. of acetone to one liter of water. This concentration was found to be non-toxic to mosquito larvae. If one or more larvae were found dead in the controls, the tests were repeated.

RESULTS

RESULTS OF PRESENT TESTS

The median lethal dose (LD50) of acetone extracts of plant products to mosquito larvae (*Culex quinquefasciatus*) are listed in Tables I and II. The values were obtained by interpolation in all cases. The LD50 values of extracts of the five most toxic plant products follow: Loblolly pine seed, 34 p.p.m., scrub pine seed, 56 p.p.m., Landino clover seed, 90 p.p.m., dusty miller (whole plant), 135 p.p.m., and elder root, 310 p.p.m. That oat seed gave an LD50 value of 1000 p.p.m. is surprising. The first sample tested came from an unknown locality; the second sample came from

TABLE I

PLANT PRODUCTS ACETONE EXTRACTS OF WHICH GAVE LD50 VALUES UP TO AND INCLUDING 1000 P.P.M. IN MOSQUITO (CULEX QUINQUEFASCIATUS) LARVICIDE TESTS

Common or trade name	Scientific name	Plant part	LD50 p.p.m.
Ambrette*	*Hibiscus abelmoschus*	Seed	1000
Astilbe	*Astilbe* sp.	Whole plant	860
Camellia	*Camellia japonica* L.	Leaves	720
Capsicum U.S.P.*	*Capsicum frutescens*	Pods	1000
Colchicum U.S.P.*	*Colchicum autumnale*	Seed	560
Devil's Shoe Strings*	*Cracca virginiana*	Roots	860
Dusty miller	*Lychnis coronaria* Desr.	Whole plant	135
Elder*	*Sambucus canadensis* or *nigra*	Root	310
Jaborandi*	*Pilocarpus jaborandi* and *P. microphyllus*	Leaves	500
Landino clover**	*Trifolium* sp.	Seed	90
Loblolly pine†	*Pinus taeda* L.	Seed	34
Mistletoe*	*Phoradendron flavescens*	Leaves	600
Oats*	*Avena sativa* N.F.	Seed	1000
Pepper, Red Bombay*	*Capsicum* sp.	Fruit	820
Rue herb*	*Ruta graveolens*	Whole plant	470
Sabadilla*	*Schoenocaulon officinalis*	Seed	450
Scrub pine†	*Pinus virginiana* Mill.	Seed	56

* Obtained from S. B. Penick & Company, New York, N. Y., and listed according to the names and spelling in their Price List and Manual of Botanical Crude Drugs, December 1, 1937, or on the package received.

** Obtained from John Harris Co., Rochester, N. Y.

† Obtained from State Tree Nursery, Jonesboro, Ill.

Iowa. It is not known whether or not the soils where these oat samples were grown are relatively free from selenium, although the second sample probably was grown in soil relatively free of selenium as poisoning from this metal is not a serious problem in Iowa.

The number of species and varieties of plants tested in this present study was 135.

DISTRIBUTION BY PLANT FAMILIES AND HIGHER GROUPS OF PRODUCTS TESTED IN THIS SURVEY

The 400 species and varieties of plants tested on mosquito larvae (*Culex quinquefasciatus*) in this and the three previous studies (24, 25, 54) fall into 102 plant families (Table III). The vast majority are flowering plants. They include species indigenous to all the continents, except Australia and Antarctica (49). The largest number of species tested in any one family was 26 for the Compositae. Of the families in which 10 or more species were tested the per cent of the species that gave LD50 values of 1000 p.p.m. or less are as follows: Pinaceae and Cucurbitaceae 33 each, Labiatae 26, Liliaceae 20, Compositae 15, Umbelliferae 14, Leguminosae 11, and Euphorbiaceae 0.

It will be noted in Table IV that four species of plants (11 per cent, in whole numbers), belonging to the class Monocotyledoneae in this study had LD50 values of 1000 p.p.m. or less, while 35 species had LD50 values greater than 1000 p.p.m. In the class Dicotyledoneae subclass Archichlamydeae 15 per cent had LD50 values of 1000 p.p.m. or less, as compared with 13 per cent for the subclass Metachlamydeae. The ratio of species containing toxic plant products to non-toxic plant products to mosquito larvae is fairly constant in the class Angiospermae. It will also be noted in Table IV that the products obtained from species of plants belonging to the higher taxonomic groups furnished a larger proportion of mosquito larvicides than did species of lower classification.

DISCUSSION

One of the objects of the present survey was to find plant materials that would serve either directly or indirectly as sources of insecticides. It was found by Harvill and others (26) that the addition of piperine, the alkaloid contained in the dry fruit of black pepper (*Piper nigrum* L.), to a pyrethrin solution gave a product that was extremely toxic to houseflies (*Musca domestica* L.). Piperine was found to be more toxic than pyrethrum to houseflies, but its paralyzing action is too slow to produce the knockdown required by fly sprays. A concentration of 0.10 per cent piperine kills 75 per cent and the pyrethrins kill 50 per cent of the flies by the Peet-Grady method. Mixtures of 0.05 per cent piperine and 0.01 per cent py-

TABLE II

PLANT PRODUCTS ACETONE EXTRACTS OF WHICH WHEN TESTED ON MOSQUITO LARVAE (CULEX QUINQUEFASCIATUS) GAVE LD50 VALUES GREATER THAN 1000 P.P.M.

Common or trade name and plant part*	Scientific name
Almond Meal	
Aloes, Curacao, U.S.P.	*Aloe vera*
Anchusa**	*Anchusa officinalis* L.
Arnica Leaves	*Arnica montana*
Arnica Root, True	*Arnica montana*
Asafoetida Gum, U.S.P.	*Ferula foetida* & sp.
Asparagus Seed	*Asparagus officinalis*
Avocado**	*Persea americana* Mill.
Balmony Leaves	*Chelone glabra*
Belladonna Leaves, U.S.P.	*Atropa belladonna*
Beth Root, N.F.	*Trillium erectum* & sp.
Birch Bark	*Betula lenta*
Black Acacia Bark**	*Robinia pseudo-acacia* L.
Black Ash Bark	*Fraxinus nigra*
Blackberry Bark of Root	*Rubus* sp.
Black Indian Hemp Root, N.F.	*Apocynum cannabinum*
Black Oak Bark	*Quercus tinctoria*
Black Walnut Bark	*Juglans nigra* L.
Black Walnut Root**	*Juglans nigra* L.
Black Willow Buds	*Salix nigra*
Bladder Wrack	*Fucus vesiculosus* & sp.
Boldo Leaves, N.F.	*Boldu boldus*
Broom Corn Seed	*Andropogon arundinaceus* var. *saccharatus*
Broom Top	*Cytisus scoparius*
Bryonia Root, White, N.F.	*Bryonia alba* & *B. dioica*
Buchu Leaves, Long, N.F.	*Barosma serratifolia*
Buckthorn Bark, N.F.	*Rhamnus frangula*
Butternut Leaves	*Juglans cinerea*
Calabar Beans	*Physostigma venenosum*
Calamus Root, Natural	*Acorus calamus*
Canella Bark	*Canella winterana*
Chickweed	*Stellaria media*
Chiretta Herb	*Swertia chirayita*
Chive**	*Allium schoenoprasum* L.
Cissus**	*Cissus sicyoides* L.
Cleavers Herb	*Galium aparine*
Cocillana Bark	*Guarea rusbyi*
Cocoa Shells	*Theobroma cacao*
Colchicum Root, N.F.	*Colchicum autumnale*
Colombo Root, N.F.	*Jateorrhiza palmata*
Condurango Bark, N.F.	*Marsdenia condurango*
Conium Leaves	*Conium maculatum*
Cranesbill Root	*Geranium maculatum*
Dogwood Bark, Jamaica***	*Ichthyomethia piscipula*
Dragon's Blood, Reeds, Thin	*Calamus draco*
Ergot, U.S.P., European, New Crop	*Claviceps purpurea*
False Bittersweet Bark	*Celastrus scandens*
Flax seed, U.S.P.	*Linum usitatissimum*
Foenugreek Seed	*Trigonella foenum-graecum*
Fraxinus**	*Fraxinus americana* L.
Grains of Paradise	*Amomum melegueta*
Horse Tail Rush	*Equisetum arvense*

Note: Products not marked by symbols were obtained from S. B. Penick & Company, N. Y., and listed according to the names and spelling on their Price List and Manual of Botanical Crude Drugs, December 1, 1937, or on the package received.

* When plant part is not specified, whole plant was used.

** Obtained from Boyce Thompson Arboretum or grown in the Institute gardens and greenhouses or collected locally.

*** Obtained through the courtesy of Dr. George Ruehle, Homestead, Fla.

TABLE II—(*continued*)

Common or trade name and plant part*	Scientific name
Irish Moss†	*Chondrus crispus* (L.) Stackh.
Jersey Tea Root	*Ceanothus americanus*
Johnson grass††	*Sorghum halepense* L.
Kalanchoe**	*Kalanchoe daigremontiana* Hamet et Perrier
Kentucky blue grass††	*Poa pratensis* L.
Knot Grass	*Polygonum aviculare*
Kola Nuts, N.F.	*Cola nitida* & sp.
Life Root Plant	*Senecio aureus*
Lycopodium, U.S.P.	*Lycopodium clavatum*
Madder Root	*Rubia tinctoria*
Mandrake Root, U.S.P.	*Podophyllum peltatum*
Masterwort Root	*Imperatoria ostruthium*
Matico Leaves	*Piper angustifolium*
Melilot Flowers, N.F.	*Melilotus officinalis*
Mezereon Bark, N.F.	*Daphne* sp.
Nettle Herb	*Urtica dioica* & sp.
Nutgalls, Aleppo, U.S.P.	*Quercus infectoria* & sp.
Oak Bark**	*Quercus* sp.
Orris Root, Florentine, N.F.	*Iris florentina*
Pimpinella Root	*Pimpinella saxifraga* or *P. magna*
Poke Root, N.F.	*Phytolacca americana*
Poplar Bark, Rossed	*Populus tremuloides*
Potato sprout**	*Solanum tuberosum* L.
Pulsatilla Herb	*Pulsatilla vulgaris* & sp.
Queen of Meadow Leaves	*Eupatorium purpureum*
Rape Seed†††	*Brassica rapus* L.
Red Top‡	*Agrostis palustris* Huds. (*A. alba* Auct.)
Rhus Aromatic Bark	*Rhus aromatica*
Rosemary Leaves	*Rosmarinus officinalis*
Rosinweed Root	*Silphium laciniatum*
Rubber plant**	*Ficus elastica* Roxb.
Sassafras Bark of Root, Natural	*Sassafras variifolium*
Simaruba Bark, Soft	*Simaruba amara* & sp.
Skunk Cabbage, Natural	*Spathyema foetida*
Snake Root, Canada, N.F., Natural	*Asarum canadense*
Squills, Red	
Stillingia Root, N.F.	*Stillingia sylvatica*
Stramonium Leaves, U.S.P.	*Datura stramonium*
Strophanthus Seed, N. F.	*Strophanthus kombe* or *S. hispidus*
Sweet Fern	*Comptonia peregrina*
Taxodium‡‡	*Taxodium distichum* L.
Thunder God Vine	
Tung Oil‡‡‡	*Aleurites fordii* Hemsl.
Tung Seeds‡‡‡	*Aleurites fordii* Hemsl.
Turpeth Root	*Operculina turpethum*
Uva Ursi Leaves, N.F.	*Arctostaphylos uva-ursi*
Wandering Jew**	*Zebrina pendula* Schnizl.
Watercress**	*Radicula nasturtium-aquaticum* (L.) Britten & Rendle
Water Pepper Herb	*Polygonum hydropiper*
Wild Turnip Root	*Arisaema triphyllum*
Willow Bark**	*Salix* sp.
Yarrow Herb	*Achillea millefolium*
Zedoary Root	*Curcuma zedoaria*

† Obtained from the sea off the coast of Maine.
†† Obtained from W. Atlee Burpee Co., Philadelphia, Pa.
††† Obtained through the courtesy of Prof. F. N. Fagan, State College, Pa.
‡ Obtained from Joseph Harris Company, Inc., Rochester, N. Y.
‡‡ Obtained from State Tree Nursery, Jonesboro, Ill.
‡‡‡ Obtained from American Tung Oil Association, Poplarville, Miss.

TABLE III

ALPHABETICAL LIST OF PLANT FAMILIES FROM WHICH ACETONE EXTRACTS OF PLANT PRODUCTS WERE TESTED ON MOSQUITO LARVAE (CULEX QUINQUEFASCIATUS)

Plant family	No. spp. & var. tested	LD50 1000 p.p.m. or less, No. spp. & var.	Plant family	No. spp. & var. tested	LD50 1000 p.p.m. or less, No. spp. & var.
Aceraceae	2	0	Malvaceae	2	1
Anacardiaceae	3	0	Menispermaceae	3	0
Apocynaceae	2	0	Myricaceae	3	0
Araceae	4	0	Myrtaceae	3	2
Aristolochiaceae	3	0	Oleaceae	2	0
Asclepiadaceae	3	0	Osmundaceae	2	0
Berberidaceae	3	0	Papaveraceae	4	1
Betulaceae	2	0	Pedaliaceae	2	1
Boraginaceae	2	0	Pinaceae	10	3
Burseraceae	2	0	Piperaceae	4	3
Caprifoliaceae	3	2	Platanaceae	2	0
Caryophyllaceae	2	1	Polygonaceae	3	0
Celastraceae	3	0	Polypodiaceae	6	1
Compositae	26	4	Ranunculaceae	8	0
Convolvulaceae	2	1	Rhamnaceae	3	0
Cruciferae	6	2	Rosaceae	8	0
Cucurbitaceae	15	5	Rubiaceae	4	0
Ericaceae	3	0	Rutaceae	5	2
Euphorbiaceae	18	0	Salicaceae	4	1
Fragaceae	5	0	Sapindaceae	3	1
Gentianaceae	2	0	Saxifragaceae	2	2
Geraniaceae	3	0	Scrophulariaceae	7	0
Gramineae	6	1	Simarubaceae	2	0
Iridaceae	3	0	Solanaceae	7	2
Juglandaceae	2	0	Sterculiaceae	2	0
Labiatae	19	5	Styracaceae	2	0
Lauraceae	6	0	Umbelliferae	14	2
Leguminosae	18	2	Zingiberaceae	4	0
Liliaceae	10	2			
Loganiaceae	2	0			
			No. families tested—one species each	42	4

rethrins were more toxic than fly sprays containing pyrethrins alone at a concentration of 0.10 per cent. Derivatives of piperine prepared by Synerholm and others (47) are equally or even more effective as fly sprays.

Acetone extracts of black pepper at concentrations of about 4000 p.p.m. failed to give satisfactory kills of *Aphis rumicis* L. Negative results were also obtained with an acetone extract of black pepper (1.2 per cent) emulsified with Penetrol (0.5 per cent) when tested as a stomach poison against the Mexican bean beetle. Satisfactory kills were obtained when bean plants were dusted with black pepper and adult bean beetles were allowed to feed on the foliage under greenhouse conditions.

Dewey (13) reports that piperine is an activator of pyrethrum dusts and is effective in the control of the German roach.

Safrole, a component of certain essential oils derived from species of Lauraceae, has been found to be an advantageous intermediate for synthesis of insecticidal compounds. Prill and others (43) have prepared a large number of compounds which are derivatives of safrole and isosafrole, a number of which were found active in fly sprays.

A steam distillate of elecampane (*Inula helenium*) gave an average kill of 89 per cent to *Aphis rumicis* at a concentration of 0.18 per cent (25). An acetone extract of the seeds and leaves of *Koelreuteria paniculata* Lamx. gave an LD50 value of 245 p.p.m. on mosquito larvae. Active extracts of plant products of several species of plants were found to vary greatly in insecticidal action. This is true of sage root (*Salvia officinalis* L.) which ranged in toxicity from an LD50 18.3 p.p.m. to greater than 1000 p.p.m. This species, for example, has not been included among the ten most toxic species tested in this survey.

TABLE IV

LIST OF PHYLA, CLASSES, AND SUBCLASSES* FROM WHICH ACETONE EXTRACTS OF PLANT PRODUCTS WERE TESTED ON MOSQUITO LARVAE (CULEX QUINQUEFASCIATUS)

Phyla, class, and subclass	No. of families	No. spp. and var.	
		LD50 1000 p.p.m. or less	LD50 greater than 1000 p.p.m.
Thallophyta	4	0	4
Pteridophyta	4	1	6
Gymnospermae	3	3	9
Angiospermae			
Monocotyledoneae	11	4	35
Dicotyledoneae			
Archichlamydeae	58	24	151
Metachlamydeae	22	19	144
Totals	102	51	349

* Classification according to Engler-Diels (15).

"Filixsäure" of Boehm (7), a compound derived from crude filicin, a product of the rhizome of male fern (*Aspidium filix-mas* [L.] Sw.), was found to be four times as toxic to mosquito larvae as crude filicin (54). The LD50 of crude filicin was estimated as 11 p.p.m. while that for Filixsäure was 2.9 p.p.m. Satisfactory control of *Aphis rumicis* was obtained with a spray containing 0.03 per cent of crude filicin, and 0.5 per cent Penetrol. When tested by the Peet-Grady method on houseflies a grade "B"[2] insecticide was obtained with a spray containing 0.05 per cent pyrethrins and 0.2 per cent crude filicin in Deo-base as a solvent.

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[2] Average knockdown 95 per cent; average kill 53 per cent.

SUMMARY

Acetone extracts of products of 400 species and varieties of plants representing 102 families were tested on mosquito larvae (*Culex quinquefasciatus* Say) under laboratory conditions in this survey. Tests were made on other species of insects only to a limited extent.

The largest number of species tested in any one plant family was 26 for the Compositae. The per cent of species that gave LD50 values of 1000 p.p.m. or less, expressed as whole numbers, follows: Pinaceae and Cucurbitaceae 33 each, Labiatae 26, Liliaceae 20, Compositae 15, Umbelliferae 14, Leguminosae 11, and Euphorbiaceae 0.

Products of the 10 species of plants, exclusive of essential oils and rotenone, possessing the greatest toxicity to mosquito larvae, expressed as median lethal dose in p.p.m., follow: balm of Gilead 5.8, male fern (filicin) 11, *Echinacea* 16, cubeb 24, black pepper 29, loblolly pine seed 34, scrub pine seed 56, Landino clover seed 90, dusty miller 135, and *Koelreuteria paniculata* Lamx. seeds and leaves 245. Acetone extracts were used in all cases, except with male fern where chemical methods other than acetone extraction were used to obtain crude filicin from the rhizome.

Satisfactory kills were obtained against the Mexican bean beetle (*Epilachna varivestis* Muls.) when bean plants were dusted with black pepper and adults allowed to feed on the foliage under greenhouse conditions. Sprays made with acetone extracts of black pepper were ineffective in controlling this insect.

A steam distillate of elecampane roots gave a satisfactory kill of *Aphis rumicis*.

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PYRETHRIN EXTENDERS

MARTIN E. SYNERHOLM, ALBERT HARTZELL, AND VICTOR CULLMANN

Pyrethrum, the insecticidally active principle of the flower heads of *Chrysanthemum cinerariaefolium* Vis., continues to hold its place of importance where insecticides exhibiting rapid action and low toxicity toward warm-blooded animals are required. Its remarkable ability to cause paralysis of certain insects within a few minutes in very low concentrations (a few hundredths of a per cent) has not been approached by any other insecticide. For this reason, pyrethrum is generally used in conjunction with the synthetic materials in compounding insecticidal sprays for uses where rapid knockdown is especially desirable.

The principal objection to the use of pyrethrum at sufficiently high concentration to effect a high kill as well as knockdown of houseflies and to ensure its effectiveness against other insect pests is its high cost. If the pyrethrins present in pyrethrum could be synthesized cheaply enough or if a relatively inexpensive substitute for pyrethrins could be found and one which is as safe to use, these would deserve widespread acceptance by insecticide users. The first of these possibilities has not materialized and the second is being realized most rapidly in the form of extenders, some of which act as synergistic agents when used with a trace of pyrethrins, showing an effectiveness equal to that of much higher concentrations of pyrethrins alone.

The most effective pyrethrin extenders in use today belong among the organic compounds possessing a methylenedioxyphenyl group. Compounds containing this group are most readily obtained from safrole, occurring in the root bark of certain members of the Lauraceae. This material and piperine, the principal alkaloidal component in the fruit of black pepper (*Piper nigrum* L.), are the most abundant methylenedioxyphenyl compounds in nature.

The first compound of this type to be used for this purpose was sesamin (3, 4) occurring in sesame oil. More recently, the isosafrole-*n*-propyl maleate condensation product ("propyl isome") (10), "piperonyl cyclohexenone" (11), and "piperonyl butoxide" (11) have been used commercially to effect a saving of pyrethrins in household sprays and aerosols.

The purpose of this report is to present insecticidal data on additional compounds and to describe methods for their preparation. This is an extension of work along the same lines that has been carried on in this labora-

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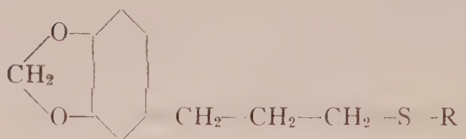
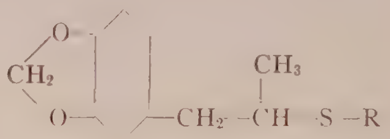
tory for the past several years. During this time Prill, Hartzell, and Arthur (8) reported on some insecticidal thio ethers prepared by the peroxide-catalyzed addition of certain mercaptans to safrole and isosafrole. The compounds with which this report is concerned are oxidation products of some of these thio ethers, namely their sulfoxides and sulfones.

CHEMICAL CONSIDERATIONS

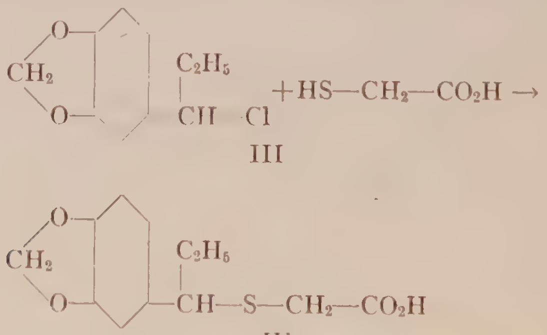
The thio ethers formed from safrole and isosafrole by the addition of a mercaptan in the presence of a peroxidic catalyst were considered by Prill *et al.* (8) on the basis of the mode of addition of mercaptans to styrene (5) to have the generic formulas I and II respectively, wherein the RS group


I


II

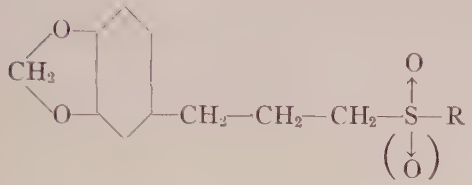
corresponds to the mercaptan used in the addition. These structures were not proved, and it seemed worthwhile to establish more conclusively the structural formulas of these thio ethers.

It has been reported (1, p. 27) that hydrogen chloride adds to isosafrole producing the very reactive 1-(3,4-methylenedioxyphenyl)-1-chloropropane III. Using thioglycolic acid as the mercaptan and this hydrochloride of isosafrole III, the thio ether IV has been prepared:

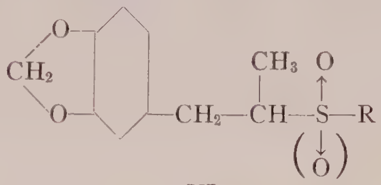

III

IV

In the direct addition of thioglycolic acid to safrole and to isosafrole, two different thio ethers are formed which are isomeric but not identical with IV. Since the position of attachment of the sulfur to the α carbon atom of the side chain in IV seems certain, assuming the accuracy of the reported mode of addition of hydrogen chloride to isosafrole (1), and because this product differs from either of the products obtained from thioglycolic acid and safrole or isosafrole by direct addition, further evidence is had that formulas I and II correctly represent the direct addition products. There is no reason to assume that thioglycolic acid would behave differently from other mercaptans, and R may be considered any radical—alkyl or aryl. With the structure of the addition products established, their oxidation products, the sulfoxides (and sulfones) may be represented by V and VI,

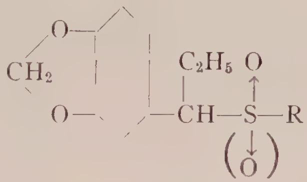


V



VI

and the corresponding oxidation products of the isosafrole hydrochloride-mercaptan product by VII. This type is of special interest because the



VII

intermediate type IV thio ethers may be readily prepared by heating the mercaptans with isosafrole in the presence of catalytic amounts of elemental iodine. This does not appear to have been reported previously in the literature. The iodine reacts rapidly with some of the mercaptan forming hydrogen iodide, which undoubtedly adds to isosafrole, and the product reacts with the mercaptan to form a thio ether of type IV, regenerating the hydrogen iodide.

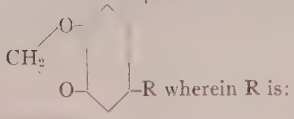
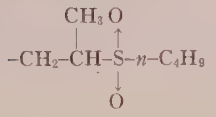
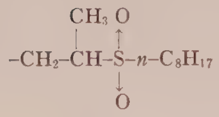
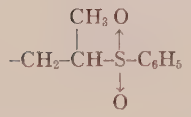
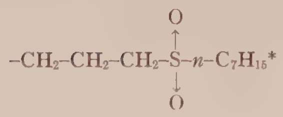
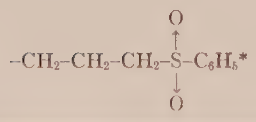
It is known (2, 5) that peroxides catalyze the addition of mercaptans to olefins. Light (2) has a similar effect; in this work, irradiation of the reaction mixture with ultraviolet light gave considerably improved yields. It is this procedure which is described and recommended here for carrying out the direct addition to obtain thio ethers of types I and II.

The sulfoxides were obtained by oxidizing the thio ethers in acetone with the equivalent amount of hydrogen peroxide. They were viscous oils with an aromatic odor reminiscent of isosafrole. The sulfones were obtained in excellent yields, usually as solids, by the use of an excess over two equivalents of hydrogen peroxide in glacial acetic acid.

The characterization of some of the solid sulfones (Table I) establishes more completely the nature of these materials. The sulfoxides are non-

TABLE I

SULFONES CORRESPONDING TO SOME THIO ETHERS FORMED IN THE PEROXIDE-CATALYZED ADDITION OF MERCAPTANS TO SAFROLE AND ISOSAFROLE

Compound  –R wherein R is:	M.p., ° C.	Sulfur, %	
		Calcd.	Found
 $-CH_2-\overset{\overset{CH_3}{\vert}}{CH}-\overset{\overset{O}{\uparrow}}{\underset{\underset{O}{\downarrow}}{S}}-n-C_4H_9$	78	11.22	11.42
 $-CH_2-\overset{\overset{CH_3}{\vert}}{CH}-\overset{\overset{O}{\uparrow}}{\underset{\underset{O}{\downarrow}}{S}}-n-C_8H_{17}$	58	9.42	9.48
 $-CH_2-\overset{\overset{CH_3}{\vert}}{CH}-\overset{\overset{O}{\uparrow}}{\underset{\underset{O}{\downarrow}}{S}}-C_6H_5$	92–93	10.52	10.52
 $-CH_2-CH_2-CH_2-\overset{\overset{O}{\uparrow}}{\underset{\underset{O}{\downarrow}}{S}}-n-C_7H_{15}*$	71–72	9.82	9.70
 $-CH_2-CH_2-CH_2-\overset{\overset{O}{\uparrow}}{\underset{\underset{O}{\downarrow}}{S}}-C_6H_5*$	75–83	10.52	10.52

*Derived from safrole.

distillable oils. There is little doubt that they are the sulfoxides inasmuch as they are formed from the thio ethers under conditions which generally are known to yield sulfoxides. Furthermore, they are easily converted to the corresponding sulfones with an additional equivalent of hydrogen peroxide.

PREPARATION OF COMPOUNDS

Intermediate thio ethers of type II. The general method is illustrated by the addition of *n*-octyl mercaptan to isosafrole. Fifty grams (0.31 mole) of isosafrole and 46 g. (0.31 mole) of *n*-octyl mercaptan were heated together at 200° for eleven hours under a type S-4 General Electric sun lamp placed six inches from the reaction flask. The mixture was distilled under reduced pressure. Forty-three grams of forerun consisting of unchanged isosafrole and mercaptan distilled below 150°/3 mm. The thio ether came over at 183 to 186°/1 mm. The yield was 55 g. (57 per cent).

Intermediate thio ethers of type IV. The general method is illustrated by the reaction between *n*-octyl mercaptan and isosafrole in the presence of iodine. Thirty-two grams of isosafrole (0.20 mole) and 28 g. of *n*-octyl mercaptan (0.19 mole) were heated together to 150° when 0.25 g. of iodine was added. The temperature rose slightly and the mixture was heated at 150 to 160° for thirty minutes. The mixture was distilled under reduced pressure. A forerun, consisting of isosafrole, *n*-octyl mercaptan and some free iodine, distilled to 160°/1 mm. The yield of thio ether boiling at 180 to 185°/2 mm. was 35 g. (60 per cent). Shaking the reaction mixture with a sodium bisulfite solution did not prevent the occurrence of iodine in the forerun.

Thioglycolic acid reacts much more readily with isosafrole in the presence of iodine. Warming on the steam bath is sufficient to complete the reaction. The product, a solid, melts at 82 to 83° and does not depress the melting point of a sample of the thio ether prepared by the reaction at room temperature of thioglycolic acid and isosafrole which has been treated with an equivalent of hydrogen chloride in the cold. This product differs from the viscous oil obtained by the direct addition of isosafrole and thioglycolic acid. It also differs from the safrole-thioglycolic acid addition product, m.p. 71° C.

Sulfoxides derived from the thio ethers. The same method of oxidation of the thio ethers to their sulfoxides was used for each type of compound. The oxidation of the *n*-octyl mercaptan-isosafrole addition product is described here as an example. Seventy-five grams (0.244 mole) of the *n*-octyl mercaptan-isosafrole adduct were dissolved in 275 ml. of acetone and 29 ml. (0.254 mole) of 30 per cent hydrogen peroxide (Merck's "Superoxol") were added. After standing eighteen hours, the acetone and finally the water were removed by heating under reduced pressure on a steam bath. The resulting oil was not purified further, but was obtained in quantitative yield as the crude product, pale yellow in color, and possessing an aromatic odor somewhat resembling the original isosafrole. It is soluble in most organic solvents except petroleum ether. It is soluble in "Deo-base" to the extent of only about one per cent. A cosolvent such as acetone may be used to prepare more concentrated solutions.

Sulfones derived from the thio ethers. The preparation of the sulfone from the *n*-butyl mercaptan-isosafrole adduct is typical. Thirty grams (0.12 mole) of the thio ether in 200 ml. of glacial acetic acid were treated with 28 g. of 30 per cent hydrogen peroxide and allowed to stand eighteen hours. The mixture was poured into water and filtered. After one recrystallization from aqueous alcohol the white, odorless solid melted at 77 to 78° *Anal.* Calcd. for $C_{14}H_{20}O_4S$: S, 11.22. Found: S, 11.42.

METHOD OF TESTING

Against houseflies (*Musca domestica* L.). Solutions of each compound to be tested were prepared by dissolving it in Deo-base (refined kerosene) to make 100 ml. of solution. In the cases where pyrethrins were added, the required amounts of a stock solution of pyrethrum extract in Deo-base were added to the samples. Adult houseflies (5 days old) of both sexes were sprayed under Peet-Grady test (7) conditions. The knockdown and kill values reported in the tables were obtained in single tests using approximately 500 flies. The compounds were compared with the O.T.I. (Official Test Insecticide), a solution of 0.1 g. of pyrethrins in 100 ml. of refined kerosene run in duplicate on the same culture of flies. The data in the tables were selected from runs in which the O.T.I. values fell within the 30 to 55 per cent kill range and were within ten units of each other. In this way the results are based on flies which are known to be of similar resistance toward pyrethrum sprays.

Acute toxicity tests on white rats. The sulfoxide obtained from the *n*-octyl mercaptan-isosafrole adduct (type VI) and the analogous type VII compound were each mixed thoroughly into ground Rockland Rat Diet at a 0.5 per cent concentration. Two rats were fed on each mixture over a three-week period. The rats ate readily of the mixture gaining from 20 to 30 g. apiece during the experiment. At the end of this time, the rats were sacrificed and an examination of their internal organs made. No abnormalities could be found when the treated animals were compared with the control. These compounds appear to possess no acute toxicity toward warm-blooded animals.

DISCUSSION OF RESULTS

The sulfoxides were compared at 0.1 per cent concentration with 0.0125 per cent pyrethrins to ensure adequate knockdown values for the Peet-Grady procedure. The knockdown values were all of the order of 90 per cent whenever pyrethrins were used. The 24-hour kill values obtained with the sulfoxides are reported in Table II.

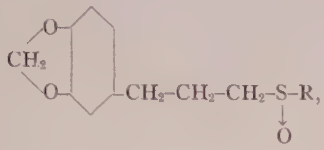
The sulfones (Table III) have an activity approximately equal to that of the sulfoxides but they have the practical disadvantage that they are less soluble in kerosene.

In Table IV some of the sulfoxides and sulfones derived from the iodine-initiated addition products are shown. Less emphasis is placed upon these because the final materials are somewhat inferior to their isomeric relatives described above.

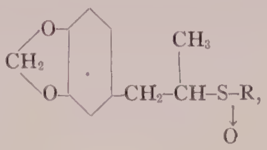
Both the sulfoxides and sulfones are superior to their intermediate thio ethers at the concentrations tested (Table V). This is interesting in view of the popular feeling that insecticidal activity is linked with lipoid solubility in a way which would predict greater activity for a more lipoid soluble

TABLE II

Insecticidal Effectiveness toward Houseflies of Certain Sulfoxides of Type V,



and Type VI,



Derived from the Peroxide-Catalyzed Addition Products of Safrole and Isosafrole, Respectively, with Mercaptans

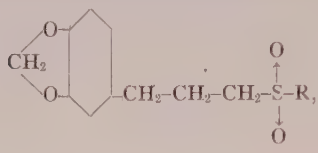
(Single tests run on approximately 500 flies using 0.1 g. of compound and 0.0125 g. pyrethrum per 100 ml. solution)

Compound wherein R is:	Type V		Type VI	
	24-Hr. kill, %	O.T.I. kill, %	24-Hr. kill, %	O.T.I. kill, %
$-C_2H_5$ (Ethyl)			28	47
$-(CH_2)_3CH_3$ (*n*-Butyl)	37	39	35	54
$-(CH_2)_5CH_3$ (*n*-Hexyl)	53	52	55	47
$-(CH_2)_6CH_3$ (*n*-Heptyl)	61	47	61	47
$-(CH_2)_7CH_3$ (*n*-Octyl)	60	54	70	47
$-(CH_2)_8CH_3$ (*n*-Nonyl)	41	47	55	47
$-(CH_2)_9CH_3$ (*n*-Decyl)			45	45
$-(CH_2)_{11}CH_3$ (*n*-Dodecyl)	19	47	45	47
$-C_6H_5$ (Phenyl)	20	36		
$-(CH_2)_2-O-C_4H_9$ (Butoxyethyl)	26	36		
$-CH_2-C_6H_5$ (Benzyl)	26	36		
$-(CH_2)_2-O-(CH_2)_2-O-C_4H_9$ 2-(2-Butoxyethoxy)ethyl	54	54		
$-\overset{\overset{CH_3}{\vert}}{CH}-(CH_2)_5CH_3$ 2-Octyl	59	55		
Pyrethrins alone	10	50	10	50

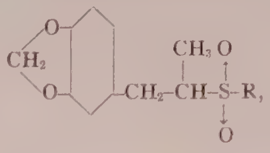
compound (all other factors remaining the same). Such a comparison, of course, can never be made, for solubility depends upon the chemical structure of the compounds as does the insecticidal activity. Here are interesting examples of cases where compounds differing only in an oxygen attached to the sulfur atom may be compared. If increasing the polarity of the molecule can be expected to impair the insecticidal effectiveness by

TABLE III

INSECTICIDAL EFFECTIVENESS TOWARD HOUSEFLIES OF CERTAIN SULFONES OF TYPE V,



AND TYPE VI,



DERIVED FROM THE PEROXIDE-CATALYZED ADDITION PRODUCTS OF SAFROLE AND ISOSAFROLE, RESPECTIVELY, WITH MERCAPTANS

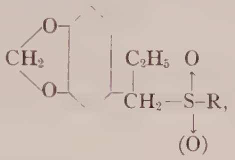
(Single tests run on approximately 500 flies using 0.2 g. of compound and 0.0125 g. pyrethrum per 100 ml. solution)

Compound wherein R is:	Type V		Type VI	
	24-Hr. kill, %	O.T.I. kill, %	24-Hr. kill, %	O.T.I. kill, %
$-CH_2CH_3$ (Ethyl)			41	46
$-(CH_2)_3CH_3$ (*n*-Butyl)	42	37	39	37
$-(CH_2)_4CH_3$ (*n*-Amyl)	39	37	49	37
$-(CH_2)_5CH_3$ (*n*-Hexyl)	51	37	65	46
$-(CH_2)_6CH_3$ (*n*-Heptyl)			76	46
$-(CH_2)_7CH_3$ (*n*-Octyl)	32	37	63	46
$-(CH_2)_9CH_3$ (*n*-Decyl)	39	44		
$-(CH_2)_{11}CH_3$ (*n*-Dodecyl)	17	44		
$-CH_2-C_6H_5$ (Benzyl)	34	50	37	50
$-CH_2-C_6H_4Cl$ (*o*-Chlorobenzyl)	20	44		
$-CH_2-C_6H_3Cl_2$ (2,4-Dichlorobenzyl)	19	44		
$-CH_2-C_6H_3Cl_2$ (3,4-Dichlorobenzyl)	28	44		
$-C_6H_5$ (Phenyl)	45	50	40	37
$-C_6H_4Cl$ (*p*-Chlorophenyl)			24	42
$-(CH_2)_2-O-C_2H_5$ (2-Ethoxyethyl)			40	50
$-(CH_2)_2-O-C_4H_9$ (2-Butoxyethyl)			21	37
$(CH_2)_2-O-(CH_2)_2-O-C_4H_9$ 2-(2-Butoxyethoxy)ethyl	50	46	50	46
Pyrethrins alone	10	50	10	50

virtue of a decrease in lipoid solubility, it is surprising that the sulfoxides and sulfones of these series are more effective than their corresponding comparatively non-polar thio ethers. The explanation very likely lies in the conclusion that the sulfoxides and sulfones owe their activity to *two* toxiphoric groups within the molecule, namely the methylenedioxyphenyl radical in each case and the sulfoxyl and sulfonyl radicals in the sulfoxides and sulfones respectively. These latter radicals have been found in other insecticidally active organic compounds: certain substituted diaryl sulfones and sulfoxides reported by Läuger, Martin, and Müller (6) to be effective stomach poisons. Also in this laboratory (9) a number of diaryl sulfones have been studied and found effective against certain agricultural pests. They are generally impractical for this use because of their marked phytotoxicity.

TABLE IV

INSECTICIDAL EFFECTIVENESS TOWARD HOUSEFLIES OF CERTAIN SULFOXIDES (AND A SULFONE) OF TYPE VII,



DERIVED FROM THE IODINE-INITIATED ADDITION PRODUCTS OF ISOSAFROLE AND MERCAPTANS

(Single tests run on approximately 500 flies using 0.25 g. of compound and 0.025 g. pyrethrum per 100 ml. solution)

Compound wherein R is:	24-Hr. kill, %	O.T.I. kill, %
$-(CH_2)_5CH_3$ (*n*-Hexyl)	80	43
$-(CH_2)_7CH_3$ (*n*-Octyl)	78	53
$-(CH_2)_{11}CH_3$ (*n*-Dodecyl)	67	45
$-(CH_2)_{11}CH_3$ (*n*-Dodecyl*)	76	45

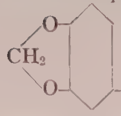
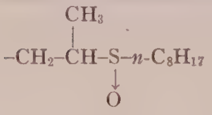
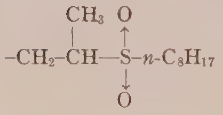
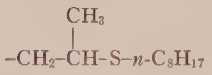
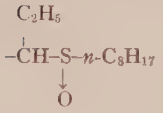
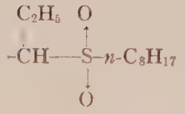
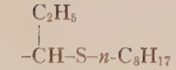
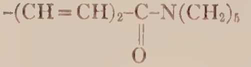
* Sulfone.

It should be emphasized that, from the standpoint of activity alone, about equally effective products are obtained from safrole and isosafrole. Safrole has the disadvantage that mercaptans add to it much more slowly than to isosafrole. Since safrole is isomerized very readily, this additional step is probably not undesirable from a practical standpoint.

TABLE V

EFFECTIVENESS AS HOUSEFLY INSECTICIDES OF CERTAIN SULFOXIDES AND SULFONES DERIVED FROM ISOSAFROLE AND MERCAPTANS COMPARED WITH THEIR THIO ETHER INTERMEDIATES AND WITH PIPERINE AND "PIPERONYL BUTOXIDE"

(Single tests using approximately 500 flies)

Compound  $-R$ wherein R is:	Concn., g. per 100 ml.	Pyrethrins, g. per 100 ml.	10-Min. knock-down, %	24-Hr. kill, %	O.T.I. kill, %
 $-CH_2-\overset{CH_3}{\overset{\vert}{CH}}-\underset{\underset{O}{\downarrow}}{S}-n-C_8H_{17}$	0.50	—	86	73	46
	0.50	0.05	99	99	46
	0.25	0.025	99	92	46
	0.125	0.0125	98	52	46
 $-CH_2-\overset{CH_3}{\overset{\vert}{CH}}-\overset{O}{\overset{\uparrow}{\underset{\underset{O}{\downarrow}}{S}}}-n-C_8H_{17}$	0.50	—	70	53	42
	0.50	0.05	93	92	42
	0.25	0.025	94	84	42
	0.125	0.0125	86	42	42
 $-CH_2-\overset{CH_3}{\overset{\vert}{CH}}-S-n-C_8H_{17}$	0.50	0.05	94	65	46
	0.25	0.025	85	53	46
	0.125	0.0125	60	24	46
 $-\overset{C_2H_5}{\overset{\vert}{CH}}-\underset{\underset{O}{\downarrow}}{S}-n-C_8H_{17}$	0.25	0.025	99	93	50
	0.125	0.0125	93	51	50
 $-\overset{C_2H_5}{CH}-\overset{O}{\overset{\uparrow}{\underset{\underset{O}{\downarrow}}{S}}}-n-C_8H_{17}$	0.50	0.05	95	94	46
	0.25	0.025	96	75	46
	0.125	0.0125	90	61	46
 $-\overset{C_2H_5}{\overset{\vert}{CH}}-S-n-C_8H_{17}$	0.50	0.05	93	63	46
	0.25	0.025	83	37	46
	0.125	0.0125	70	24	46
 $-(CH=CH)_2-\underset{\underset{O}{\Vert}}{C}-N(CH_2)_5$ (Piperine)	0.50	—	80	67	47
	0.25	0.025	98	91	51
	0.125	0.0125	98	65	53
"Piperonyl butoxide"*	0.50	—	85	44	46
	0.50	0.05	99	97	46
	0.25	0.025	96	85	46
	0.125	0.0125	96	51	46
Pyrethrins alone	—	0.05	93	32	50
	—	0.025	84	21	50
	—	0.0125	82	10	50

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* Commercial preparation supplied by Dodge and Olcott, Inc., 180 Varick St., New York 14, N. Y.

SUMMARY

New pyrethrin extenders are described with data on their effectiveness when used against houseflies (*Musca domestica* L.). These are the sulfoxides and sulfones produced by oxidation of the mercaptan-safrole and -isosafrole addition products.

The sulfoxides and sulfones are of approximately equal effectiveness, and are more active than their parent thio ethers at the concentrations tested.

The structures of the intermediate thio ethers are more completely established.

The methods of preparing these materials are described.

Some of these materials are shown to possess no toxicity toward white rats when incorporated in their diet over a three-week period.

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SEMI-MICRO METHOD FOR MEASURING THE AMOUNT OF OXYGEN CONSUMED AND CARBON DIOXIDE PRODUCED IN PLANT RESPIRATION

F. E. Denny

When it is desired to measure simultaneously the oxygen consumed and the carbon dioxide produced in the respiration of plants, the procedure first proposed by Magness and Diehl (4), or some modification of it (1, 2, 3, 5), has been found to be convenient and accurate. By this method the O_2 removed from the air surrounding the tissue is replaced continuously by the automatic addition of an equal volume of O_2 from an adjacent container of this gas, a water siphon replacing the O_2 with water.

The principal use of the method has been with bulky tissues, such as apple fruits, potato tubers, sweet potato roots, etc., and as containers for the tissue, desiccators (4 to 6 liters capacity) have in the main been used. The tissues and containers have required a considerable amount of space, an especially undesirable feature if they are to be immersed in a water bath. Possibly for large organs these large samples and containers are necessary, but with smaller organs, such as germinating seeds, small fruits, radishes, cut tissues of various kinds, etc., dependable samples can be obtained with the use of a smaller volume, and consequently a smaller container can be used. This in turn makes possible the use of a smaller oxygen-supply vessel.

As containers for these smaller tissues, fruit jars of the type with wide opening were found convenient, and in at least one respect were superior to the desiccators commonly used, in that there was available a proportionately larger area of the alkali solution for absorbing the CO_2 produced, and, furthermore, the tissue was brought nearer to the surface of this liquid. Johnstone (3) introduced a modification (not since, however, adopted by others) by which the O_2 supply cylinder was immersed in the water bath, the water level of the bath itself being used for establishing a siphon. A still further improvement was described (1) in which the overflow water was brought into the O_2 supply tube from the bottom rather than from the top. In the present experiments the measuring cylinder usually employed as a container for the O_2 supply was replaced by a bulb such as the leveling bulb in gas analytical work. After being fitted with a piece of glass tubing which reached from the bottom nearly to the tip, this bulb was immersed in the water bath, and when adjusted so that the tip of the inner tube was on a level with the water surface in the bath, water was brought into this bulb from the water bath as fast as O_2 was transferred to the vessel con-

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taining the respiring tissue. This furnished a measure of the amount of O_2 consumed. Back titration of the alkali solution in the bottom of the fruit jar showed the amount of CO_2 produced.

In this way the area of a water bath necessary for a single determination was not larger than about 100 sq. in. and could be as low as about 30 sq. in. Tests were made with such tissues as wheat seeds, *Sorbus* fruits, radish, carrot, and beet roots, potato tubers (small size), and the sample sizes ranged from about 14 to 500 grams.

When tests with samples of varying size of the same tissue were run simultaneously, a straight line relationship between the amount of tissue and the O_2 consumed (or CO_2 produced) was obtained, and the intercept of this line on the y-axis did not deviate significantly from zero.

If a large number of tests are to be made, a water bath would furnish insufficient space, but the present method can be carried out in tanks, tubs, or half-barrels in a constant temperature room, and many more determinations can be made in the same space than is possible with the standard procedure.

It is called a semi-micro method because it is unsuitable for very low rates of respiration. One should have enough tissue in a sample to cause a rate of oxygen consumption amounting to at least about one cc. of O_2 per hour. If the rate is sufficiently slow and there occurs a rapid fall in the barometric pressure, the meniscus will fall below the water level, and a reading cannot be made conveniently.

METHODS AND MATERIALS

The method. The essential features of the method are shown diagrammatically in Figure 1. The bulb (Fig. 1 A) contains oxygen, and is immersed in the tank of water until the water in the inner tube has risen to the level of the water in the bath, as shown by the meniscus, which should be flat not rounded at the time connection is made to the jar (Fig. 1 B) by means of the capillary tube (Fig. 1 C). The tissue is placed on the shelf and screen in the jar into the bottom of which a measured amount of NaOH or KOH (approximately 2 N) has been pipetted. As fast as the O_2 is removed from the air surrounding the tissue it is replaced by O_2 from the bulb, and water is siphoned over from the bath into the bulb. At the end of a test the bulb (Fig. 1 A) is removed and the accumulated water is poured into a measuring cylinder and the volume noted. If the water bath has glass sides the calibrated O_2 supply tube (Fig. 1 D) functions in a similar manner, and allows readings to be made at intervals during the course of a test. The details of adjusting the level of the O_2-bulb to the proper place in the bath, and of holding it there, of manipulating the jar containing the tissue, of computing the results so that the volume of water siphoned over can be transferred into terms of the volume of oxygen consumed, are dealt with in subsequent paragraphs.

Apparatus. In these experiments an attempt was made to utilize only such apparatus as would ordinarily be found in a plant physiological laboratory, in the catalogs of laboratory supply companies, in local stores, or could be made easily from materials commonly at hand. The adjustment of the O_2-bulb (Fig. 2 E) in the water bath so that the meniscus in the inner tube is just on a level with the water in the bath is very important, and the scheme shown in Figure 2 F, G, H, J, was found satisfactory. The bar (Fig. 2 F) is a piece of half-inch hollow pipe, smoothed by rubbing with emery cloth, and greased with either vaseline or axle-grease. Rough adjustment is made first with the lower clamp (Fig. 2 G) to bring the meniscus nearly to the water level. Then the tension of the two clamps, H and J, is slightly lessened, and by pressure on the top of the bar the level of the

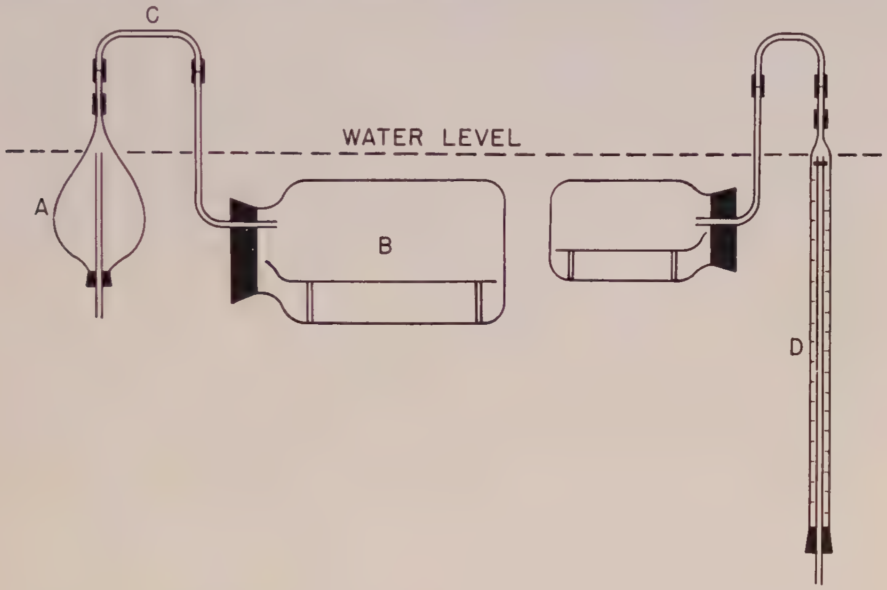

FIGURE 1. Diagram showing the essential features of the method. The bulb, A, contains oxygen and is immersed in the water until the meniscus at the top of the inner tube is level with the water surface of the bath. The plant tissue is placed in B on a screen supported by a shelf, and into the bottom of the container a measured amount of alkali is pipetted. When temperature equilibrium is attained the capillary tube, C, is inserted. As O_2 is used up in B, and as the CO_2 escaping from the tissue is absorbed, O_2 passes from A to B, and water is drawn into A from the water bath. At the end of a test, the accumulated water in the bottom of A is poured off and measured; this value, when taken in conjunction with the volumes in A and B and the barometric pressures at the start and the end, furnishes a measure of the amount of O_2 consumed. Back titration of the alkali in the bottom of B shows the amount of CO_2 produced. D is a calibrated glass tube which operates on the same principle, and, if the water bath is fitted with a window, will show the rate at chosen intervals.

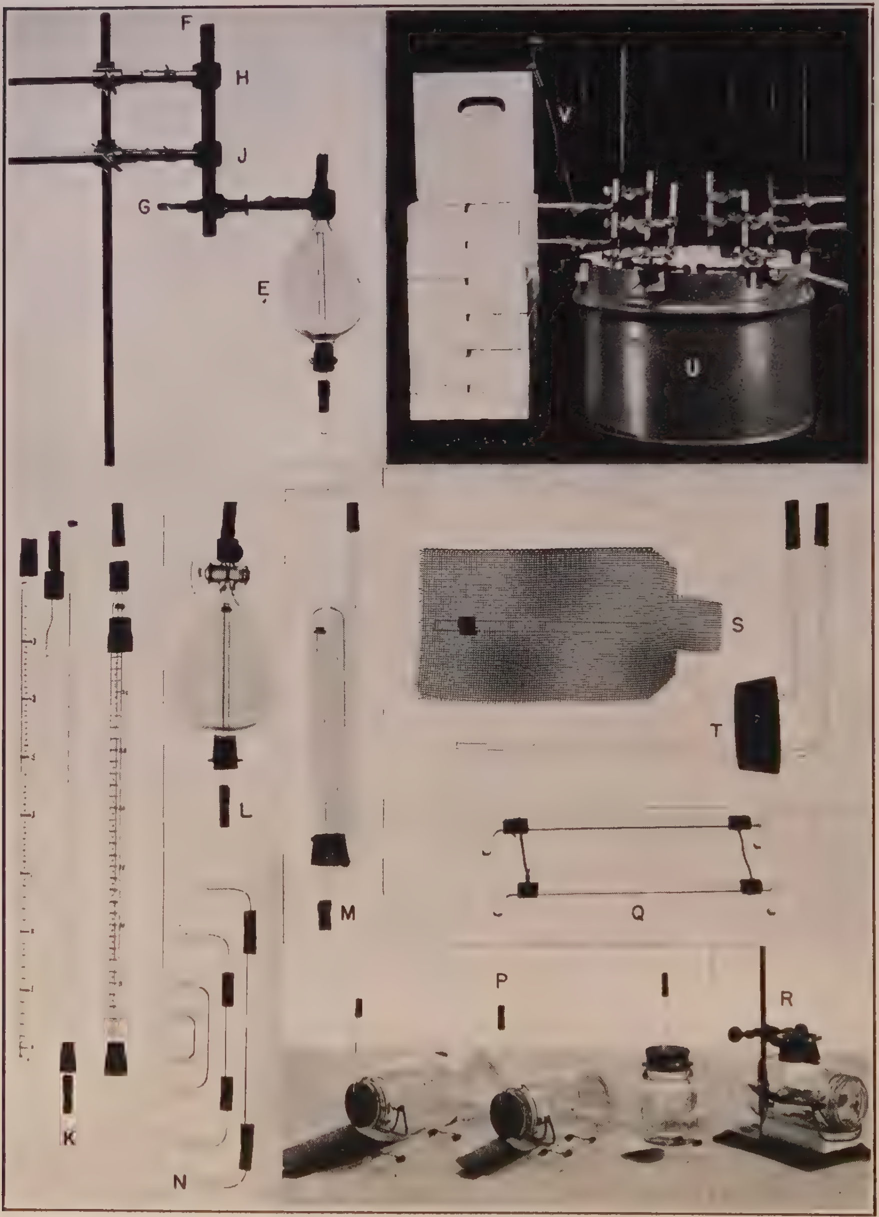

FIGURE 2. Apparatus used in measuring respiration by the "semi-micro" method. For description see text.

meniscus can be adjusted with considerable accuracy. Then the two clamps, H and J, are tightened firmly. The bulb (Fig. 2 E) is listed in commercial catalogs usually as a leveling bulb for use in gas analysis. It has a relatively wide bore at the tip so that there is little tendency for water to collect in the bore, and this permits a free adjustment of the pressure in establishing the height of the meniscus. A piece of heavy-walled tubing about the width of the clamp serves for applying the clamp firmly. The pieces of rubber tubing at the top and bottom are of lighter but firm rubber. The bulb (Fig. 2 E) is shown as it appears after it has been filled with oxygen and is ready for immersion in the bath. The upper and lower openings are closed with pieces of glass rod. The O_2 with which it is filled should be at the temperature of the bath, or below. When lowering the bulb into the bath the glass rod which seals the bottom of the tube is removed and water rises partially in the inner tube. The bulb is then pushed down into the bath until the tip of the inner tube is about one-half inch above the water level. The final adjustment of the meniscus is not made until all other preparations involving the tissue and its container have been made, and the time has arrived to connect the O_2-bulb and the container by means of the capillary connection. Then the glass rod in the upper part of the bulb is removed and the adjustment of the meniscus is made.

The bulb, Figure 2 E, is obtainable in sizes ranging from volumes of about 100 to 500 cc., the 300 cc. size being the one mainly used in these tests. Although this type of container for the O_2 supply was found preferable, other pieces of apparatus, such as those shown in Figure 2 K, L, M, were used. An ordinary separatory funnel, such as Figure 2 L, functions well but has one disadvantage: care must be taken that the bore in the stop-cock is not clogged with a drop of water; this puts a constriction on the gas pressure and interferes with the adjustment of the meniscus. Figure 2 M shows how an O_2 supply vessel may be made from a 100 cc. test tube and some glass tubing. It is necessary in this case, also, to be certain that a drop of water does not get into the U-shaped tubing which is to convey the O_2 from the vessel to the jar containing the tissue. To prevent this, the U-shaped tube is removed and its place taken by a piece of glass rod. Then the apparatus is filled with O_2 by water displacement, and while O_2 is being passed through the U-shaped tube, the glass rod is removed and the U-shaped tube is pushed into position.

The details of the calibrated tubes are shown in Figure 2 K. The upper part is made from the bulb of a 10 cc. pipette cut in two. The ink used in the calibrations is water-proof, or at least will serve satisfactorily for a few weeks under water. The ink is made as follows: to some shellac solution add enough Crystal Violet (Coleman and Bell, Norwood, Ohio) to give a good color, and thin to the proper consistency with methyl alcohol.

Figure 2 N shows some of the U-shaped capillary tubes used to make

the connection between the O_2-bulb and the container holding the tissue.

As containers for the tissue, fruit jars of the wide-mouth type were found convenient. The size of opening is the same for 2-quart, 1-quart, and pint jars, and is fitted snugly with a No. 13 rubber stopper. This equipment is shown in Figure 2 P. The 2-quart and 1-quart sizes are placed on their sides for use, but the pint size must be used in the upright position. When the alkali solution is placed in the jars, 25 or 50 cc. in the 2-quart jar, 25 cc. in the 1-quart and pint size, a good-sized absorbing layer of alkali is obtained, about 50 to 115 sq. cm. Shelves on which to place the tissue (see Fig. 2 Q) were made from pieces of glass rods, bent so as to furnish legs, and held together with No. 18 copper wire. Flexible wire screens (14 mesh, 30 BS wire), shown in front of the fruit jars in Figure 2 P, were cut with a width somewhat larger than the opening in the jar. A flap in the front part of the screen can be bent upward after the tissue is placed in the jar, and this prevents the tissue falling into the alkali. The maximum capacity of a 2-quart jar is about 500 g. of such tissues as fruits or fleshy roots. Small materials such as wheat seedlings or *Sorbus* fruits were tied in cheesecloth bags. Figure 2 R shows a convenient clamp for the jars so that they could be put into the bath and removed easily from it; the jar rests on a piece of cellulose sponge, and after a large rubber stopper is placed on top, the clamp is pressed down and fastened. A small rake, made by cutting off a 12-inch piece of three-quarter inch flat steel, turning down a half-inch of it at the end, and covering this tip with rubber tubing, was found convenient in putting tissue into and removing it from the container.

In these experiments the tissue and the jars were brought to the temperature of the bath before they were placed in it. If this cannot be done, the following procedure was found convenient. To the center of the top side of the wire screen fasten a piece of 1 cm. glass tubing (with an internal diameter of at least 7 mm.), see Figure 2 S. Pass a properly bent second piece of 5 mm. glass tubing through the No. 13 rubber stopper (as shown in Figure 2 T), this piece being long enough to reach well toward the back of the jar. The tissue sample is then placed in the jar, the rubber stopper being inserted in such a manner that the long member of the 5 mm. tube passes inside the tubing that is fastened to the screen. Immerse the jar in the bath and draw a current of air free of CO_2 through the jar, the temperature of the air being brought to that of the bath by passing it through a coil of copper or glass tubing that has been placed in the bath. The air current should be brisk enough to avoid any accumulation of CO_2 in the jar. When it has been found by test that the jar and contents have reached the temperature of the bath, remove the jar and stopper, and insert the alkali (previously brought to the proper temperature). This equipment (Fig. 2 S, T) may also be used in filling the containers with gas mixtures.

The use of tanks, or troughs, etc. The usual type of constant temperature bath would be suitable only if a few samples were to be tested. With a large number of samples a constant temperature room would be desirable. In such a case the apparatus shown in Figure 2 U when set up inside a constant temperature room was found satisfactory. It consists of one-half of a steel barrel (22.5 inches in diameter, 15 inches deep) fitted with an overflow pipe bent downward as shown so as to facilitate the establishment of a uniform water level with no fluctuations in this level. The water level was maintained by the siphon made from capillary-tubing (see Fig. 2 V), which transferred water drop by drop from the pail to the tank, five gallons of water being sufficient for an overnight run. Only the O_2 supply bulbs were immersed in this tank, the containers for the tissue being immersed in vessels of water placed alongside the tank. Space was available for six samples for each tank, and with some slight modifications eight or more samples could be accommodated. In these tests two such tanks were used. If a large number of tests were to be made probably a better set-up in a constant temperature room would be to use two adjacent troughs, one about 8 inches wide and about 18 inches deep into which the O_2 supply bulbs are to be immersed and in which a constant water level is maintained; and the other trough about 14 inches wide and 9 inches deep for immersing the tissue containers, this trough not needing a constant water level.

Oxygen consumed. Since it appears that the details of the method of computing the amount of oxygen consumed in an experiment of this type have not been published, an example is given herewith. Let us take the experiment shown in line 1, Table II. The data were:

Start, 9:07 a.m., barometer = 758.0 mm.
Stop, 2:17 p.m., barometer = 756.5 mm.
Temperature = 31° C.
Volume of container in excess of shelf and screen = 885 cc.
" " alkali solution = 25 cc.
" " tissue = 247 cc.
" " O_2 supply tube = 181 cc.
Net vol. of air in container = 885 − (247 + 25) = 613 cc.
Vol. of gas in connection with tissue at start = 613 + 181 = 794 cc.

This vol. of gas at 0° and 760 mm. = $\frac{273}{273+31} \times \frac{758.0}{760} \times 794 = 711$ cc.

H_2O found in O_2 supply tube at end = 69 cc.
Vol. of O_2 in O_2 supply tube at end = 181 − 69 = 112 cc.
Vol. of gas in connection with tissue at end = 613 + 112 = 725 cc.

This vol. of gas at 0° and 760 mm. = $\frac{273}{273+31} + \frac{756.5}{760} \times 725 = 648$ cc.

O_2 used up during 5.17 hrs. = 711 − 648 = 63 cc. at 0° and 760 mm.

O_2 used up during 5.5 hrs. = $\frac{5.5}{5.17} \times 63 = 67$ cc. at 0° and 760 mm.

CO_2 produced. At the end of a test the alkali in the bottoms of the tissue containers was rinsed thoroughly into a volumetric flask, enough of a saturated solution of barium chloride was added to precipitate the carbonate completely and, after making up to the mark and allowing the precipitate to settle, aliquots were taken for titration against 0.25 N HCl. The difference between this value and that of the blank obtained with the same amount of the alkali solution was taken as a measure of the amount of CO_2 produced.

Tissues. The tissues used were: tubers of potato (*Solanum tuberosum* L.), variety Irish Cobbler; roots of beet (*Beta vulgaris* L.), variety Detroit Dark Red, radish (*Raphanus sativus* L.), variety Scarlet Globe, and carrot (*Daucus carota* L. var. *sativa* DC), variety Early Golden Bell; fruits of *Sorbus aucuparia* L.; seeds of wheat (*Triticum aestivum* L.), variety Leap's Prolific. These were all grown in the Institute gardens except the wheat which was purchased from a seedsman. The tubers, roots, and fruits were stored at 20° C. for 2 to 5 days after harvest before being used in the experiments, and small sizes were selected for these tests. The wheat seeds were tied in cheesecloth bags, 7 g. in each bag, and were soaked in water for 24 hours previous to use; they were then dipped into a suspension of Wettable Spergon (1 g. to 100 cc. of H_2O), the excess liquid being removed by shaking; the weights given are the air dry weights before soaking. The fruits of *Sorbus aucuparia* were likewise tied in cheesecloth bags, 50 g. per bag.

RESULTS

The results of tests in which a comparison could be made between the rates found by the present "semi-micro" method and by the previously described method (1, p. 422), using 6-liter desiccators, are shown in Table I. The sample sizes bear definite ratios to each other, and by multiplying the "semi-micro" data by the appropriate factor, and comparing the value with that obtained by the "macro" method, it is seen that the two methods are in good agreement. The ratios of the "semi-micro" to the "macro" values range from 0.92 to 1.05, with an average close to 1.0.

Further results with the semi-micro method are shown in Table II. The values for carrot, beet, and *Sorbus* in Table II show good agreement among duplicates, and also that the rate found was approximately proportional to the amount of tissue taken.

It is the data for radish roots and wheat seedling in Table II, however, which furnish a critical test of the method. In these cases, the size of the sample was varied over a range, and the relationship between respiration rate and the size of the sample was established. The two experiments with radish were carried out with the same samples, the data for lines 15 to 26 being for the first day and that of lines 27 to 38 being for the same samples

TABLE I

COMPARISON OF RESULTS WITH "MACRO" AND "SEMI-MICRO" METHODS WITH DIFFERENT TISSUES

Tissue	Method	Hrs.	Grams of tissue	Respiration rate 20° C., cc. of gas at 0° C., 760 mm.	
				O_2 consumed	CO_2 produced
Wheat seedlings	Macro	24	64	284	256
	Semi-micro	"	32	145	119
	"	"	16	66	63
	"	"	8	35	31
Beet roots	Macro	19	1722	568	544
	"	"	"	565	542
	Semi-micro	"	287	95	90
	"	"	"	97	91
	"	"	"	94	95
	"	"	"	*	92
	"	"	"	97	92
	"	"	"	92	90
Beet roots	Macro	23	1000	323	334
	"	"	"	325	334
	Semi-micro	"	500	159	160
	"	"	"	155	153
Potato tubers	Macro	23	1500	118	122
	"	"	"	122	117
	Semi-micro	"	500	42	40
	"	"	"	42	40

* Determination lost because siphon overflowed.

on the succeeding day. The weights given are fresh weights, and the samples were re-weighed on the second day to take into account the small shrinkage of weight which occurred. The graphs giving the data for the radish roots are shown in Figure 3, from which it is seen that an approximately straight line relationship was obtained between the amount of tissue taken and the respiration rate, and furthermore, that this line passes approximately through the origin of coordinates. That the fluctuations shown by the data are the result mainly of differences in the samples and not in errors of the method is indicated by the behavior of the pairs of samples in the vicinity of 225 g. and 300 g. on the two successive days. These samples are out of line somewhat, but the same general relation between them holds for both days.

The results with the wheat seedlings are shown in Table II, 9 lines at bottom, and in Figure 4. The agreement, with the straight line relationship was much better with the wheat than with radish presumably because more uniform samples were obtained. The smallest weight of wheat used (14 g. air dry previous to soaking) was represented by about 400 grains of

TABLE II

RESULTS OF TESTS OF "SEMI-MICRO" METHOD WITH VARIOUS TISSUES

Tissue	Temp., ° C.	Hrs.	Wt. of tissue, g.	Cc. at 0° C., 760 mm.	
				O_2 consumed	CO_2 produced
Carrot roots	31	5.5	250	67	67
			"	76	74
			"	61	56
			"	57	63
Beet roots	31.5	7	130	36	39
			260	74	73
			390	116	119
			520	149	152
Sorbus fruits	20	41	50	35	41
			"	33	42
			100	73	81
			"	69	77
			150	100	117
			"	105	128
Radish roots	20	17	370	201	190
			318	178	171
			274	168	157
			235	119	115
			202	122	118
			173	96	92
			149	84	83
			128	70	65
			109	60	58
			95	54	55
			82	38	38
			70	36	41
Radish roots	20	17	362	194	190
			310	166	161
			267	163	160
			230	113	116
			200	108	113
			168	90	90
			144	82	81
			124	64	67
			105	55	56
			92	52	56
			78	35	41
			68	38	39
Wheat seedlings	20	17	14	29	28
			21	46	44
			28	55	55
			35	76	73
			42	91	83
			49	103	101
			56	121	115
			63	134	128
			70	154	137

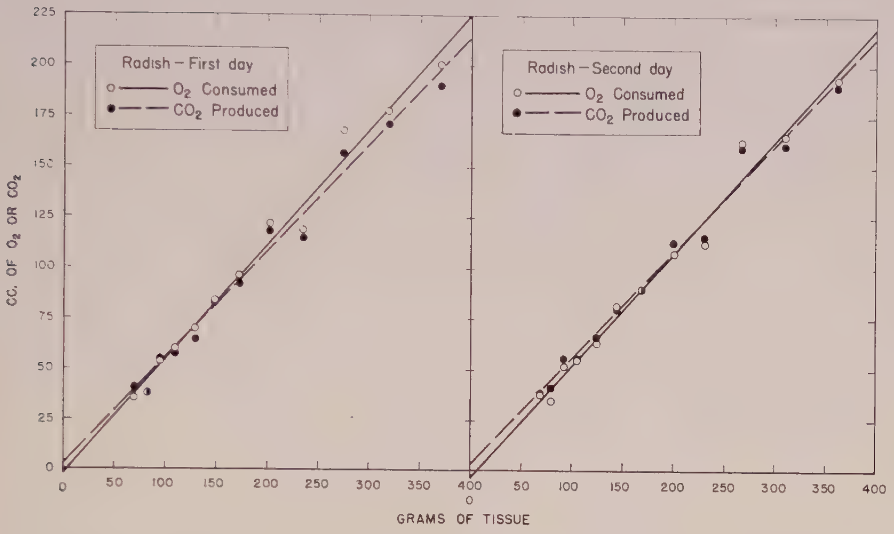

FIGURE 3. Respiration measurements with radish roots. Results of two successive days with the same samples, account being taken of a small decrease in the fresh weight in each sample due to shrinkage. Regression lines are straight, and pass close to the origin of coordinates.

wheat. With wheat, as with the radish, the regression lines pass approximately through the zero point.

Regression equations of the form: $y=a+bx$, were fitted to the data, and the constants of the equations for the lines in Figures 3 and 4 are as follows:

For radish, 1st day, O_2 consumed: $y=-2.35+0.56881x$
CO_2 produced: $y=1.78+0.52681x$
For radish, 2nd day, O_2 consumed: $y=-2.03+0.55138x$
CO_2 produced: $y=3.50+0.52512x$
For wheat, O_2 consumed: $y=-2.41+2.1976x$
CO_2 produced: $y=1.29+1.9905x$

Although the regression lines cut the y-axis at near the origin, they do not do so exactly, the intercept not being zero in any case, but varying from -2.41 to $+3.50$. The question arises whether the divergence from zero is significant.

Youden (6) has recently referred to this problem and has furnished methods of obtaining the constants of the regression equation, and of testing their significance. The details of the computations are shown for the radish data (first day) in Table III, Youden's scheme being in the main followed, although certain unimportant modifications in the form of the equations were introduced.

The three right-hand columns in Table III serve as checks on the ac-

TABLE III

METHOD OF COMPUTATION FOR ESTABLISHING THE LINE OF REGRESSION, AND FOR TESTING WHETHER THE y-INTERCEPT DIFFERS SIGNIFICANTLY FROM ZERO

x=Wt. of sample, g.	$y=O_2$ consumed, cc.	x^2	y^2	xy	Checks		
					Computed Y	$(y-Y)$	$(y-Y)^2$
370	201	136900	40401	74370	208.11	− 7.11	50.5521
318	178	101124	31684	56604	178.53	− 0.53	.2809
274	168	75076	28224	46032	153.50	14.50	210.2500
235	119	55225	14161	27965	131.32	−12.32	151.7824
202	122	40804	14884	24644	112.55	9.45	89.3025
173	96	29929	9216	16608	96.05	− 0.05	.0025
149	84	22201	7056	12516	82.40	1.60	2.5600
128	70	16384	4900	8960	70.46	− 0.46	.2116
109	60	11881	3600	6540	59.65	0.35	.1225
95	54	9025	2916	5130	51.69	2.31	5.3361
82	38	6724	1444	3116	44.29	− 6.29	39.5641
70	36	4900	1296	2520	37.47	− 1.47	2.1609
Sum 2205	1226	510173	159782	285005	1226.02	− 0.02	552.1256
Mean 183.75	102.2						

$$S.S.\,x = 510173 - \frac{(2205)^2}{12} = 105004.25 \qquad S.S.\,y = 159782 - \frac{(1226)^2}{12} = 34525.7$$

$$S.P.\,xy = 285005 - \frac{(2205)(1226)}{12} = 59727.5$$

$$\text{Slope} = b = \frac{S.P.\,xy}{S.S.\,x} = \frac{59727.5}{105004.25} = 0.56881$$

Intercept $= a = \bar{y} - b\bar{x} = 102.2 - (0.56881)(183.75) = -2.35$
Regress. Eq.: $Y = -2.35 + 0.56881\,x$; (See Col. 6.)
If s = the standard deviation of a single determination, and s_a = the standard deviation of a, then:

$$(n-2)s^2 = (y-Y)^2 = S.S.\,y - \frac{(S.P.\,xy)^2}{S.S.\,x} = 34525.7 - \frac{(59727.5)^2}{105004.25} = 552.1$$

$$10s^2 = 552.1;\ s = \sqrt{\frac{552.1}{10}} = 7.431$$

$$s_a = s\sqrt{\frac{\Sigma x^2}{n\Sigma x^2 - (\Sigma x)^2}} = 7.431\sqrt{\frac{510173}{(12)(510173) - (2205)^2}} = 4.73$$

$$t_a = \frac{2.35}{4.73} = 0.50;\ \text{D.F.} = n - 2 = 10;\ \text{Required } t_{.05} = 2.228$$

curacy of the computation. The sum of the entries in column 6 (1226.02) should equal the sum of those in column 2 (1226). The differences between the observed values in column 2 and those computed from the regression equation in column 6 are entered in column 7, and the sum of the squares of these differences (552.1256), in column 8, should equal the value opposite the entry $(n-2)s^2$, (552.1).

A summary of the values for "s", "a", "s_a" and "t_a" is given in Table IV. The "t_a" values are found by dividing the entries in column 4 by those in column 5. There were $n-2=10$ degrees of freedom for the radish tests, and the required "t" value for odds of 19 to 1 is 2.228, while for the wheat data there were available $9-2=7$ degrees of freedom, the required "t" value being in this case 2.365. Thus, no significant deviation from zero is

TABLE IV

TESTS OF THE SIGNIFICANCE OF THE DEVIATION FROM ZERO OF THE y-INTERCEPT

Tissue	Gas	s	a	s_a	t_a
Radish 1st day	O_2 CO_2	7.431 6.824	−2.35 +1.78	4.73 4.33	0.50 0.41
Radish 2nd day	O_2 CO_2	7.473 6.637	−2.03 +3.50	4.72 4.19	0.43 0.84
Wheat	O_2 CO_2	2.449 2.349	−2.41 +1.29	2.06 1.97	1.17 0.65

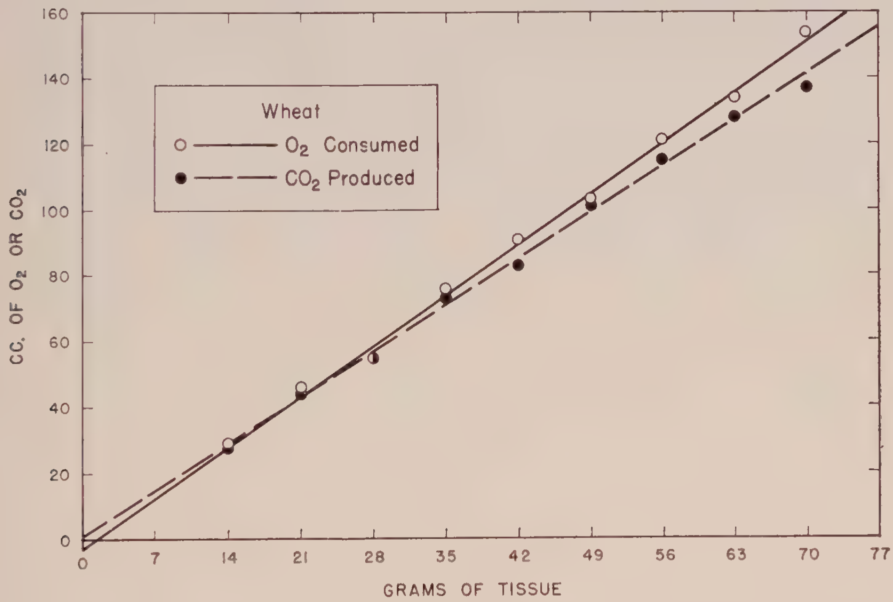

FIGURE 4. Respiration measurements with wheat seeds. Seeds soaked 24 hours before being placed in the respirometer. Smallest sample contained about 400 seeds, largest about 2000 seeds. Straight line relationship between amount of tissue and respiration rate is shown, and the regression line cuts the y-axis near the zero point.

shown for the *y*-intercept in any of the tests. The data in column 3 for "*s*" are included, since they may be of some interest in showing the size of the error term found in these experiments, "*s*" being the standard deviation of a single determination.

SUMMARY

Apparatus is described for the simultaneous measurement of the amount of oxygen consumed and carbon dioxide produced in plant respiration, and the results obtained with it on seeds, small fruits, or other organs of small size are given. The minimum quantity of tissue with which dependable results were obtained was about 14 g. of soaked wheat seeds (air dry weight before soaking), 50 g. of fruits of *Sorbus aucuparia*, and 75 g. of radish roots. Economy of space in a water bath was obtained, the area required for a single test not being more than about 100 sq. in.; and for still smaller organs it could be as low as about 30 sq. in. Suitable equipment for use with a large number of samples in a constant temperature room is described. When samples of different amounts of the same tissue were used, a straight line relationship between the amount of tissue taken and the amount of O_2 consumed (or CO_2 produced) was obtained, and the *y*-intercept of this regression line did not differ significantly from zero. The method is unsuitable for tissues with very low rates of respiration. The sample taken should cause a rate of oxygen consumption amounting to at least about one cc. of O_2 per hour.

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EFFECT UPON PLANT RESPIRATION CAUSED BY CHANGES IN THE OXYGEN CONCENTRATION IN THE RANGE IMMEDIATELY BELOW THAT OF NORMAL AIR

F. E. Denny

Although the effect on respiration resulting from the use of different concentrations of oxygen has been tested over a wide range, there have been very few measurements in the interval from about 15 per cent O_2 to normal air content, about 20.9 per cent.

Yet, as indicated from a previous consideration of some of the pertinent literature (3), it is particularly in this portion of the oxygen curve that tests are needed, not merely to establish more completely the points on the oxygen concentration curve, but especially to make possible a decision as to the credibility of many measurements of respiration which have already been made. For if a decrease in O_2 in this range influences the rate of respiration, then the validity of measurements that have been made under conditions bringing about the gradual decrease in O_2 below that of normal air content is called into question. And furthermore, if the reverse is true, that a considerable variation in O_2 content is permissible in this range without any effect upon respiration, then studies of the effect of CO_2 upon respiration, which necessarily involve simultaneous changes in O_2 concentration, will be facilitated.

It is with regard to the effect of changes in oxygen concentration in this rather narrow range that these experiments have been carried out. The question was: If the O_2 concentration is maintained at a constant level in the range just below 20.9 per cent, at what concentration can a definite change in respiration rate be established?

In a previous article (3) approximately the same range of O_2 concentrations was investigated but under conditions for the continuous depletion of the O_2 content of the surrounding air. In the present tests each chosen O_2 concentration was maintained at that level throughout the test.

The employment of O_2 concentrations that would produce a distinctly large and easily recognized effect was avoided. Instead, those that appeared to be just at the margin at which an effect could barely be shown were sought.

Tests were made with tubers of potato, roots of radish, beet, and turnip, and fruits of tomato. In general, it may be stated that with the

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first four named above, decreases in respiration rate were obtained when the O_2 concentration was reduced from 20.9 per cent to about 15 to 16 per cent but that the amount of this decrease was small, approximately 5 per cent. A reduction to 18 per cent O_2 was not followed by a significant change in the rate either of oxygen consumption or carbon dioxide production.

The tests with tomato fruits showed no decrease even with 15 per cent O_2, but reasons are given for the view that the method employed was unsuitable with this particular tissue, and that the results should be regarded merely as inconclusive.

MATERIALS AND METHOD

Tissues. The plants were grown in the Institute gardens and the organs used were: tubers of potato (*Solanum tuberosum* L. var. Irish Cobbler); roots of radish (*Raphanus sativus* L. var. Burpee's Red Giant); roots of turnip (*Brassica rapa* L. var. White Egg); roots of beet (*Beta vulgaris* L. var. Good-for-all); and fruits of tomato (*Lycopersicon esculentum* Mill. var. Pomodoro). The tissues were stored at 20° C. for 2 to 5 days after harvest before being placed in the respiration vessels.

Apparatus. As containers for the tissue, glass desiccators with a capacity of approximately six liters were used. These were of the bowl type without a conical construction at the base, the shelf resting on lugs near the bottom. The alkali solution used for absorbing the CO_2 was approximately 2N NaOH, 100 cc. being placed in a 15 cm. evaporating dish in the bottom of the desiccator immediately below the shelf. The details of the apparatus used for continuously supplying oxygen to the vessels to replace that used up in respiration, and for maintaining a constant water level by means of which a measurement of the amount of oxygen consumed could be made, are given in a preceding paper (3, p. 422–427). The amount of CO_2 produced was estimated by titration of the alkali solution in the bottom of the desiccator. All tests were made in a constant temperature room regulated at 20° C. by thermostatic control. The desiccators containing the tissue were immersed in vessels of water as a further precaution against temperature fluctuations.

Oxygen concentrations. The desired oxygen concentrations were prepared by water displacement in an inverted 5-gallon bottle equipped with inlet and outlet tubes, one extending the length of the bottle and the other only merely through the stopper. Graduation marks on this bottle showed the levels to which oxygen and nitrogen were to be brought to give the desired volume percentage of the two gases. It was found necessary to insure that the bottle was level at the time of the final adjustment of gases to the calibration marks. These prepared gas mixtures were then stored at approximately the temperature of the respiration room (20° C.) until time for their transfer into the desiccators containing the tissue. Through the hole in the cover of the desiccator inlet and outlet glass tubes with short rubber connections for flexibility were inserted, the inlet tube reaching through the hole in the shelf to a point just below the shelf, and the outlet tube reaching just below the top stopper. A roll of wire screen placed vertically over the hole in the shelf held the sample of tissue away from this central core, and allowed the cover with its inlet tube to be fitted on even when the desiccator was nearly full of tissue. When the time arrived for the start of a test, the alkali solution was placed in the evaporating dish in the bottom of the desiccator, the screen core was placed over the hole in the shelf, the tissue was put in the desiccator, and the cover was put in place. Then the outlet from the 5-gallon bottle was connected with the inlet of the desiccator, and the inlet of the 5-gallon bottle was connected with the tap-water faucet. The entire gas sample in the 5-gallon bottle was pushed into the desiccator in about 4 minutes. The desiccator with its sample of tissue was then immersed in the vessel of water in the respiration room, and was connected to the graduated glass cylinder containing the oxygen. The constant water level device then pushed oxygen gas into the desiccator as fast as it was used up in respiration, and so the percentage of oxygen prepared and transferred to the desiccator at the start was maintained throughout the test at approximately the desired value. Some tests were made to determine to what extent the desired percentage was actually attained. This was done by removing samples of the air in the desiccator at the end of a test and analyzing the gas for oxygen by gas analytical methods. For example in lines 1 and 5 in Table I the desired percentage of O_2 was 20.9 and the percentages found at the end of the tests were: 19.8, 21.6, 21.0, 21.3, 20.3, 21.1, 20.7, 20.6, av. 20.8; in lines 2 and 4, the desired value was 16.0, and the values found were: 16.9, 15.0, 16.1, 15.4, 17.8, 15.5, 16.1, 15.6, av. 16.1. The data for lines 7 and 11 were: wanted 20.9, found 21.3, 22.3, 19.1, 20.7, 20.5, 21.3, 19.9, 20.1, av. 20.6; and for lines 8 and 10 the values were: wanted 18.0, found 19.8, 18.5, 18.1, 17.4, 18.3, 18.7, 18.5, 17.8, av. 18.4. Thus, while some deviations were found, it is believed that the agreement between wanted and found values was sufficiently good for the purposes of the experiment.

The gas mixtures of O_2 and CO_2 were made from cylinders of compressed gases, and a few tests were run to compare such mixtures with normal air. Using beet roots, four samples of each type of gas gave: air, 347, 345, 339, 346 cc. of O_2 consumed; gas mixture to give 20.9 per cent O_2, 322, 333, 371, 337.

RESULTS

In Table I are shown the respiration rates, i.e. cc. of O_2 consumed and CO_2 produced, in the various tests, and a summary of the data in Table I is given in Table II, which shows the amount of the change in rate found

TABLE I

RESPIRATION RATE OF SAMPLES RECEIVING DIFFERENT CONCENTRATIONS OF OXYGEN

Tissue, weight, time	Period	% O_2	Cc. O_2 consumed						Cc. CO_2 produced					
			Replicate samples				Totals	Mean	Replicate samples				Totals	Mean
Potato tubers 2250 g., 24 hrs.	1	20.9	491	466	483	482	1922		502	463	461	470	1896	
	2	16.0	466	454	466	457	1843		446	458	465	465	1834	
		Diff.	−25	−12	−17	−25	− 79	−19.75	−56	− 5	+ 4	− 5	− 62	−15.50
	1	16.0	446	434	456	427	1763		447	447	459	444	1797	
	2	20.9	480	479	472	457	1888		471	470	482	484	1907	
		Diff.	+34	+45	+16	+30	+125	+31.25	+24	+23	+23	+40	+110	+27.50
Potato tubers, 2250 g., 24 hrs.	1	20.9	464	481	514	493	1952		472	491	510	498	1971	
	2	18.0	471	475	520	496	1962		491	493	530	502	2016	
		Diff.	+ 7	− 6	+ 6	+ 3	+ 10	+ 2.50	+19	+ 2	+20	+ 4	+ 45	+11.25
	1	18.0	458	470	497	496	1921		464	478	498	506	1946	
	2	20.9	458	484	521	481	1944		465	484	517	514	1980	
		Diff.	0	+14	+24	−15	+ 23	+ 5.75	+ 1	+ 6	+19	+ 8	+ 34	+ 8.50
Potato tubers, 1000 g., 74 hrs.	1	20.9	241	255	240	244	980		245	256	248	251	1000	
	2	18.0	218	254	242	223	937		226	255	235	227	943	
		Diff.	−23	− 1	+ 2	−21	− 43	−10.75	−19	− 1	−13	−24	− 57	−14.25
	1	18.0	248	238	245	251	982		239	239	248	245	971	
	2	20.9	226	227	224	214	891		231	232	238	241	942	
		Diff.	−22	−11	−21	−37	− 91	−22.75	− 8	− 7	−10	− 4	− 29	− 7.25
Radish roots, 1500 g., 7 hrs.	1	20.9	310	330	296	303	1239		302	321	296	301	1220	
	2	12.0	252	261	247	240	1000		265	279	255	258	1057	
		Diff.	−58	−69	−49	−63	−239	−59.75	−37	−42	−41	−43	−163	−40.75
	1	12.0	278	288	266	270	1102		283	290	273	279	1125	
	2	20.9	270	296	270	267	1103		279	300	274	278	1131	
		Diff.	− 8	+ 8	+ 4	− 3	+ 1	+ 0.25	− 4	+10	+ 1	− 1	+ 6	+ 1.50
Radish roots, 1647 g., 7 hrs.	1	20.9	317	265	322	317	1221		302	304	298	288	1192	
	2	16.0	272	277	275	272	1096		253	261	254	257	1025	
		Diff.	−45	+12	−47	−45	−125	−31.25	−49	−43	−44	−31	−167	−41.75
	1	16.0	313	313	307	285	1218		301	303	295	264	1163	
	2	20.9	280	296	293	258	1127		265	276	273	237	1051	
		Diff.	−33	−17	−14	−27	− 91	−22.75	−36	−27	−22	−27	−112	−28.00
Radish roots, 500 g., 18 hrs.	1	20.9	197	197	192	199	785		206	195	192	223	816	
	2	15.0	194	194	194	196	778		189	189	181	192	751	
		Diff.	− 3	− 3	+ 2	− 3	− 7	− 1.75	−17	− 6	−11	−31	− 65	−16.25
	1	15.0	198	194	198	184	774		201	196	189	185	771	
	2	20.9	207	207	208	189	811		200	197	205	189	791	
		Diff.	+ 9	+13	+10	+ 5	+ 37	+ 9.25	− 1	+ 1	+16	+ 4	+ 20	+ 5.00
Tomato fruits 1500 g., 17 hrs.	1	20.9	340	331	331	343	1345		373	362	360	380	1475	
	2	18.0	335	348	327	335	1345		350	366	340	346	1402	
		Diff.	− 5	+17	− 4	− 8	0	0.00	−23	+ 4	−20	−34	− 73	−18.25
	1	18.0	342	350	320	318	1330		373	387	355	355	1470	
	2	20.9	373	342	317	321	1353		404	361	327	340	1432	
		Diff.	+31	− 8	− 3	+ 3	+ 23	+ 5.75	+31	−26	−28	−15	− 38	− 9.50

TABLE I—(*Continued*)

Tissue, weight, time	Period	% O_2	Cc. O_2 consumed						Cc. CO_2 produced					
			Replicate samples				Totals	Mean	Replicate samples				Totals	Mean
Tomato fruits, 1500 g., 17 hrs.	1	20.9	411	412	419	399	1641		419	419	423	410	1671	
	2	15.0	358	357	379	372	1466		390	383	407	416	1596	
		Diff.	−53	−55	−40	−27	−175	−43.75	−29	−36	−16	+ 6	− 75	−18.75
	1	15.0	430	402	424	414	1670		452	416	445	429	1742	
	2	20.9	357	362	366	395	1480		372	379	390	421	1562	
		Diff.	−73	−40	−58	−19	−190	−47.50	−80	−37	−55	− 8	−180	−45.00
Beet roots, 1480 g., 18 hrs.	1	20.9	267	274	278	278	1097		279	284	272	292	1127	
	2	18.0	250	240	252	253	995		238	243	249	257	987	
		Diff.	−17	−34	−26	−25	−102	−25.50	−41	−41	−23	−35	−140	−35.00
	1	18.0	267	280	269	280	1096		282	285	274	283	1124	
	2	20.9	247	260	248	264	1019		244	253	248	266	1011	
		Diff.	−20	−20	−21	−16	− 77	−19.25	−38	−32	−26	−17	−113	−28.25
Beet roots, 1474 g., 18 hrs.	1	20.9	267	275	267	271	1080		264	273	265	270	1072	
	2	15.0	244	260	243	260	1007		245	263	258	254	1020	
		Diff.	−23	−15	−24	−11	− 73	−18.25	−19	−10	− 7	−16	− 52	−13.00
	1	15.0	266	265	268	278	1077		261	265	268	268	1062	
	2	20.9	262	256	263	262	1043		260	273	268	262	1063	
		Diff.	− 4	− 9	− 5	−16	− 34	− 8.50	− 1	+ 8	0	− 6	+ 1	+ 0.25
Beet roots, 1400 g., 17 hrs.	1	20.9	269	275	290	281	1115		277	282	297	299	1155	
	2	15.0	249	256	272	267	1044		248	249	262	267	1026	
		Diff.	−20	−19	−18	−14	− 71	−17.75	−29	−33	−35	−32	−129	−32.25
	1	15.0	258	288	271	261	1078		271	292	282	275	1120	
	2	20.9	272	278	282	262	1094		267	277	274	271	1089	
		Diff.	+14	−10	+11	+ 1	+ 16	+ 4.00	− 4	−15	− 8	− 4	− 31	− 7.75
Turnip roots, 1000 g., 16 hrs.	1	20.9	584	546	533	505	2168		552	519	499	484	2054	
	2	18.0	552	528	496	490	2066		520	498	471	460	1949	
		Diff.	−32	−18	−37	−15	−102	−25.50	−32	−21	−28	−24	−105	−26.25
	1	18.0	526	506	550	575	2157		497	493	524	546	2060	
	2	20.9	519	488	529	555	2091		490	457	498	527	1972	
		Diff.	− 7	−18	−21	−20	− 66	−16.50	− 7	−36	−26	−19	− 88	−22.00
Turnip roots, 1000 g., 17 hrs.	1	20.9	521	536	521	533	2111		509	503	473	498	1983	
	2	15.0	463	481	445	471	1860		441	452	421	444	1758	
		Diff.	−58	−55	−76	−62	−251	−62.75	−68	−51	−52	−54	−225	−56.25
	1	15.0	458	474	500	538	1970		444	446	475	503	1868	
	2	20.9	439	444	486	523	1892		416	416	454	492	1778	
		Diff.	−19	−30	−14	−15	− 78	−19.50	−28	−30	−21	−11	− 90	−22.50

in each test, and whether this change was significant as compared with the experimental error found in that particular test.

To make clear how these computations were made let us take as an illustration the data in lines 1 to 6 in Table I, and lines 1 and 2 in Table II. The first six lines in Table I show the result of a comparison of the two O_2 concentrations, 20.9 per cent and 16.0 per cent. Line 1 shows that the four samples which received 20.9 per cent O_2 during the first period of 24 hours consumed 491, 466, 483, and 482 cc. of O_2, and line 2 shows that when, the next day, these same four samples were given 16.0 per cent O_2 the rates were 466, 454, 466, and 457. Line 4 shows that the four lots which received 16.0 per cent O_2 the first day consumed 446, 434, 456, and 427 cc. of O_2, and on being transferred the next day to 20.9 per cent O_2, consumed 480, 479, 472, 457 cc. of O_2. Line 3, column 9 shows that the four samples in lines 1 and 2 decreased in rate by an average of 19.75 cc. of oxygen per sample on being transferred from 20.9 per cent O_2 to 16 per cent O_2. Then, line 6 shows that the other four samples gained an average of 31.25 cc. per sample on being transferred from 16.0 per cent to 20.9 per cent. On the face of it, it appears that 20.9 per cent O_2 has given a higher rate of O_2 consumption than has 16.0 per cent O_2, but the significance of the difference is not readily apparent. This is determined by computing the *s* value, i.e. the standard deviation of a single determination, and a *t* value, i.e. the ratio obtained by dividing the difference between the means of the two O_2 concentrations undergoing comparison by the standard error of this difference. Brandt (1) has furnished the details of the methods available for making such a test of significance. An example of such a computation is shown in the footnote to Table II for the potato data in the first six lines of Table I.

The discussion so far has related to the values for oxygen consumed. The values for the carbon dioxide produced simultaneously by the same samples are shown in columns 10 to 15 in Table I, and the same methods were applied.

A summary of such computations made on the data in Table I is shown in Table II. The entries of greatest interest are of course those in columns 4 and 8 showing the percentage increase or decrease in the respiration rate by changing the O_2 concentration from 20.9 per cent to some lower value, and the entries in columns 6 and 10 showing the *t* value for each comparison from which an estimate can be made as to the significance of the observed difference. In each experiment there were available six degrees of freedom for the error term, i.e. three from each set of four replicate samples, and from a *t* table (4, p. 65) it is found that for probabilities of 0.05 and 0.01 the *t* values should be at least 2.447 and 3.707 respectively.

The potato tuber data in lines 1 and 2, Table II, show that reducing the O_2 concentration to 16.0 per cent O_2 from 20.9 per cent definitely reduced the rate of both O_2 consumption and CO_2 production, although the extent of this reduction was not large being 5.4 per cent for O_2 consumption and 4.5 per cent for CO_2 production. Two tests, however, comparing 20.9 per cent with 18.0 per cent O_2, lines 3 to 6, one for a 24-hour interval and the other for a 74-hour period, did not show differences in rate due to this change in O_2 concentration.

TABLE II

SUMMARY DERIVED FROM THE DATA OF TABLE I

Tissues and time	% O_2	Cc. O_2 consumed (0° C., 760 mm.)				Cc. CO_2 produced (0° C., 760 mm.)			
		Total	% Incr. or decr.	*s*	*t*	Total	% Incr. or decr.	*s*	*t*
Potato, 24 hrs.	20.9 16.0	3810 3606	−5.4	9.61	7.51**	3803 3631	−4.5	20.20	3.01*
Potato, 24 hrs.	20.9 18.0	3896 3883	−0.3	12.92	0.36	3951 3962	+0.3	8.64	0.45
Potato, 74 hrs.	20.9 18.0	1871 1919	+2.5	11.96	1.42	1942 1914	−1.4	7.24	1.37
Radish, 7 hrs.	20.9 12.0	2342 2102	−10.2	7.83	10.83**	2351 2182	−7.2	4.66	12.82**
Radish, 7 hrs.	20.9 16.0	2348 2314	−1.4	21.36	0.56	2243 2188	−2.5	6.80	2.86*
Radish, 18 hrs.	20.9 15.0	1596 1552	−2.8	2.94	5.28**	1607 1522	−5.3	9.35	3.21*
Tomato, 17 hrs.	20.9 18.0	2698 2675	−0.9	14.75	0.55	2907 2872	−1.2	22.56	0.55
Tomato, 17 hrs.	20.9 15.0	3121 3136	+0.5	18.87	0.28	3233 3338	+3.2	25.10	1.48
Beet, 18 hrs.	20.9 18.0	2116 2091	−1.2	5.16	1.71	2138 2111	−1.3	8.68	1.10
Beet, 18 hrs.	20.9 15.0	2123 2084	−1.8	5.89	2.34	2135 2082	−2.5	5.64	3.32*
Beet, 17 hrs.	20.9 15.0	2209 2122	−3.9	7.91	3.89**	2244 2146	−4.4	4.08	8.49**
Turnip, 16 hrs.	20.9 18.0	4259 4223	−0.8	8.81	1.42	4026 4009	−0.4	9.27	0.65
Turnip, 17 hrs.	20.9 15.0	4003 3830	−4.5	8.37	7.31**	3761 3626	−3.6	8.27	5.77**

* Significant at the 0.05 level of probability; ** at the 0.01 level.

Note: Computations for *s* and *t* values for the potato data in lines 1 to 6 in Table I are as follows:

Sum of squares, line $3=(-25)^2+(-12)^2+(-17)^2+(-25)^2-(-79^2\div 4)=123$

Sum of squares, line $6=(34)^2+(45)^2+(16)^2+(30)^2-(125^2\div 4)=431$

$$s=\sqrt{\frac{123+431}{(4-1)+(4-1)}}=9.61;\quad t=\frac{31.25-(-19.75)}{9.61}\sqrt{\frac{(4)\ (4)}{4+4}}=7.51$$

Total O_2 consumed: by 20.9% $O_2=1922+1888=3810$; by 16% $O_2=1843+1763=3606$. Reduction in rate due to 16% $O_2=204$, which is 5.4% of the rate with 20.9% O_2.

With radish roots, lines 7 to 12, the first two tests were with duration periods of only seven hours, 12 per cent O_2 showing reductions in rate, amounting to 10.2 per cent for O_2 consumption and 7.2 per cent for CO_2 production. The comparison between 20.9 per cent O_2 and 16.0 per cent O_2 showed no lowering of the rate of O_2 consumption, but CO_2 production showed a small (2.5 per cent) reduction which was significant, at least at the 0.05 level of probability. The test with radish was repeated over an 18-hour interval using 15.0 per cent O_2, lines 11 and 12, and in this case reductions in rate below the base rate with 20.9 per cent O_2 were shown for both O_2 consumption and CO_2 production. The reductions, however, were again small being only 2.8 per cent for O_2 consumption and 5.3 per cent for CO_2 production. Perhaps the result for O_2 consumption may be regarded as somewhat doubtful on account of the unusually low *s* value (2.94) associated with this test, but the *s* value for the CO_2 production (9.35) was acceptable without doubt.

The tomato fruit results are shown in lines 13 to 16, and no significant reduction in the rate of respiration was shown by the use of either 15 or 18 per cent O_2. It is believed, however, that the results with tomato are inconclusive, not merely because the *s* values were high, but because the use of the switchback or reversal type of experiment was of no advantage in the tomato fruit tests. As will be discussed in a later paragraph, the advantage of this type of experiment depends upon whether the values of a given set of samples show a positive correlation between the first and subsequent periods of measurement. The tomato values do not show such a correlation. Dependence must then be placed on random sample variation for an error term, and in this case this term has been too high for an efficient test. The tomato fruits were in the transition stage between a green and a red color, and presumably at this stage the rates of ripening vary considerably from fruit to fruit, which may account for the lack of correlation between the behavior of a sample in the two successive periods of observation.

The values for the tests with beet roots are shown in lines 17 to 22. No significant differences in rates between 20.9 and 18.0 per cent O_2 were found. Two tests comparing 20.9 and 15.0 per cent O_2 were carried out. In one the *t* value for O_2 consumed was beyond the 0.01 level but in the other it did not quite reach the 0.05 point; for CO_2 consumed the 0.01 level was exceeded in one test, and was nearly reached in the other. However, the decreases in rate due to the decrease in O_2 concentration from 20.9 to 15.0 per cent O_2 were small, amounting to only 1.8 and 3.9 per cent for O_2 consumed, and to 2.5 and 4.4 per cent for CO_2 produced.

The results with turnip roots are shown in the four lines at the bottom of Table II. Reducing the O_2 concentration to 18.0 per cent did not definitely decrease the rate either of O_2 consumed or CO_2 produced, but

15.0 per cent O_2 retarded the rate of O_2 consumption by 4.5 per cent, and that of CO_2 consumed by 3.6 per cent.

Considering the data as a whole, it appears that a reduction of the O_2 concentration to 15.0 per cent has retarded the respiration rate (either O_2 consumed or CO_2 produced) by about 5 per cent. A reduction of the O_2 concentration to 18.0 per cent did not show a retardation but in most cases the change, although small and not significant by the *t* test, was in the negative direction, indicating that the beginning of the effective range in retarding respiration was approximately at this point.

DISCUSSION

The advantage that may be obtained by the use of the switchback or reversal type of experiment, such as that used in these experiments, depends upon the occurrence of a positive correlation between the values shown by a given set of replicate samples in the successive periods of observation. That is, a sample showing a high rate in the first period should show a high rate in the succeeding period if an advantage by this method is to be obtained. If such a correlation does not occur no reduction in sample error has been accomplished.

Correlation coefficients were computed for the data in Table I (omitting the tomato values), i.e. between the four sets of replicates in lines 1 and 2, 4 and 5, 7 and 8, etc. for both O_2 consumed and CO_2 produced, using the method shown by Chambers (2, p. 43). The twenty-two correlation coefficients so obtained for each of the two series (O_2 consumed and CO_2 produced) were then combined (2, p. 55). The resulting correlation coefficients were 0.844 for O_2 consumed, and 0.841 for CO_2 produced, highly significant values for 25 degrees of freedom. The correlation coefficients for the tomato data were computed separately, the following values being found: for O_2 consumed −.161, .743, .084, −.329; for CO_2 produced, −.209, .588, −.483, −.270.

In one respect the results of the present experiment do not correspond with those of the previous test (3) in which the effect of a reduction in oxygen concentration upon respiration rate was measured. In the previous series the O_2 surrounding the tissue was not maintained at a chosen concentration but was allowed to become depleted progressively down to 15 per cent O_2, or below. The results indicated that the effect of the reduced oxygen supply was much greater upon O_2 consumption than upon CO_2 production. This difference in the response of the two phases of respiration was not observed in the present tests, the effect being about the same upon CO_2 production as upon O_2 consumption. No explanation for this difference in the two sets of experiments has been found.

However, both the present and the previous tests may be regarded as favoring the view that changes in the O_2 concentration in this region from about 15 per cent to 20.9 per cent O_2 have had rather small effects quantitatively, and furnish assurance that respiration measurements previously made under conditions for the gradual reduction in the oxygen supply are probably dependable. If the O_2 supply did not fall below 18 per cent, the error due to change in O_2 concentration may be regarded as negligible, and even if it fell to a value of around 16 per cent O_2, only small effects upon respiration would have been introduced by the concomitant fall in the O_2 concentration.

SUMMARY

The effect upon both oxygen consumption and carbon dioxide production resulting from decreasing the oxygen content of the surrounding air from that of normal air (about 20.9 per cent) to about 15 per cent by volume was determined.

When the O_2 content was maintained at about 15 per cent, the respiration rate of potato tubers, and roots of radish, beet, and turnip was depressed, but only by about 5 per cent below that of the controls receiving 20.9 per cent O_2. No reduction in rate was established when the change was from 20.9 per cent to 18.0 per cent O_2, but there was evidence that this is a marginal concentration below which some reduction in rate may be expected. With tomato fruits no depression in rate of respiration was observed even with 15 per cent O_2, but results with this tissue were regarded as inconclusive.

Measurements of such small differences as occurred in these tests were dependent upon the use of the switchback or reversal type of experiment in which the tissue samples were divided into two series, one receiving the two different oxygen concentrations in a given order, while the other series received the two concentrations in the reverse order. A significant positive correlation between the values for the two successive periods was observed (except in the case of the tomato fruits), this resulting in a low experimental error for the comparison of differences between treatments.

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SOME IMPROVEMENTS IN EQUIPMENT FOR EVALUATING FUNGICIDES BY THE FOLIAGE DISEASE METHOD

S. E. A. McCallan

In 1943 a method of evaluating fungicides by means of tomato foliage diseases was described, together with the equipment employed (2). Since then various improvements have been made and because of the interest in this development a brief description seems desirable.

The equipment is now housed in a basement laboratory measuring 28×23 feet. The room is illuminated by skylights and fluorescent lighting. Placed in the center of the laboratory are three incubation chambers, and along the walls are work benches, tables, sink and hood.

Incubation chambers. The incubation chambers have been improved by insulation and temperature control and are shown in Figure 1. These are of the following internal dimensions, 6 feet across the front (one foot more than before), 5 feet high and 3 feet deep. They are constructed of Flexboard, an asbestos composition board, enclosing a 2-inch insulating core of sheet cork. Only the top and door are of glass, where two sheets are used with an inch air space between. For convenience, and as before, the chambers are set on legs 18 inches above the floor. Humidity control is maintained as before by the atomizing jet nozzles[1] situated at the center of each end 6 inches below the top. However, due to the condensation of water vapor by the refrigeration coils, it is necessary that the humidity nozzles be turned on continuously while the chamber is in use in order to maintain a relative humidity of 100 per cent. Limited information indicates that a 100 per cent relative humidity is at least equal in importance to the optimum temperature for obtaining maximum disease. The temperature is controlled in two chambers by a York system employing Freon 12 refrigerant. There are three sets of coils in each chamber situated on each side and back wall just under the plate glass protection shelf. The back coil measures 48 inches across by 20 inches down, and the side coils 24 by 20 inches. The third insulated chamber used primarily for weathering tests (3) has no temperature control machinery.

In order to ascertain the desirable operating temperatures for the chambers it was necessary to determine the incubation and infection temperature responses for the diseases of interest. Three tomato (*Lycopersi-*

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[1] Turbo Humidifier Part 6A13, parts size No. 8. Parks-Cramer Co., Fitchburg, Mass.

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con esculentum Mill.) plants 6 to 7 inches tall were nioculated with spores of *Alternaria solani* (Ell. & Mart.) Jones & Grout or *Phytophthora infestans* (Mont.) de Bary (potato strain) and held under individual bell jars at each of the following temperatures: 5°, 10°, 12.5°, 15°, 18°, 21°, 24°, 27°, 30°, and 35° C. These tests were repeated. The weighted average results, adjusted to a maximum of 1000 spots per plant (3 leaves), are shown in Figure 2. It will be seen that the optimum temperature for late blight infection is approximately 17° C. and for early blight 20° C. However, within a range of 3° C. on either side, infectivity is not less than 90 per cent of maximum. It has been shown elsewhere (1) that the optimum temperature for infection of snapdragon (*Antirrhinum majus* L.) with *Puccinia antirrhini* Diet. & Holw., is approximately 13° C. with an even wider range of about 5° C. on either side before there is a reduction of 10 per cent in number of pustules. Thus a temperature of 18° C. could be expected to give reasonably good infection results for all three diseases. However, since two temperature-controlled chambers were available, one was set at 21° C.,

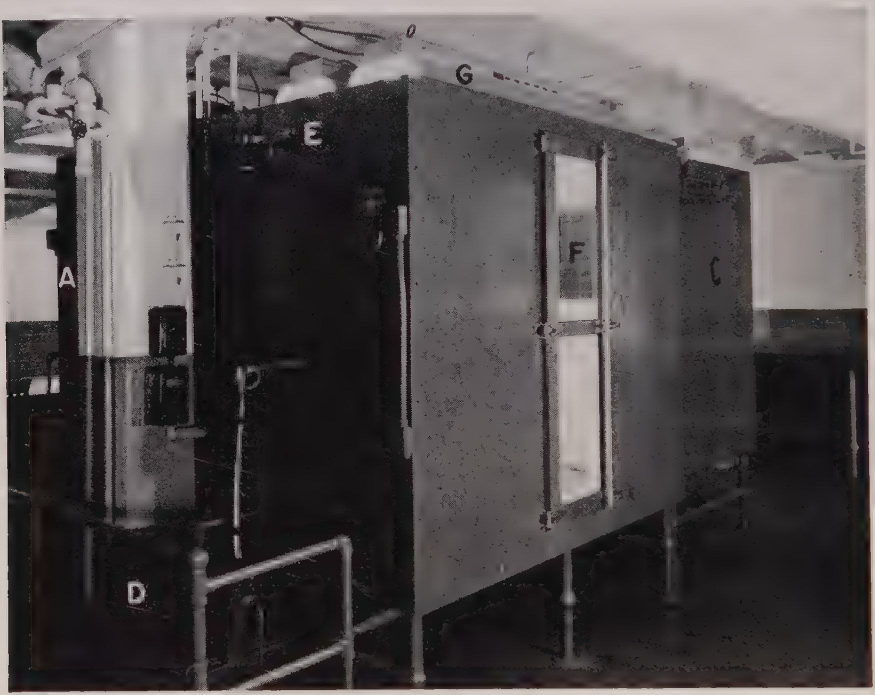

FIGURE 1. Insulated incubation chambers A, B, and C. Equipment for chamber B: refrigeration machine D, humidifying nozzle with air pressure and water lines E, fluorescent lights G, and portion of refrigeration coils at F.

i.e. 70° F., for early blight and the other at approximately 15° C., i.e. 60° F., for late blight and snapdragon rust. Since it has been found more efficient to run early and late blight tests at the same time, that is, to spray a double set of plants, it is also desirable to have two different infection chambers for these two diseases. Ordinarily all three diseases are given a 24-hour "incubation period" in the chambers before removal to the greenhouse.

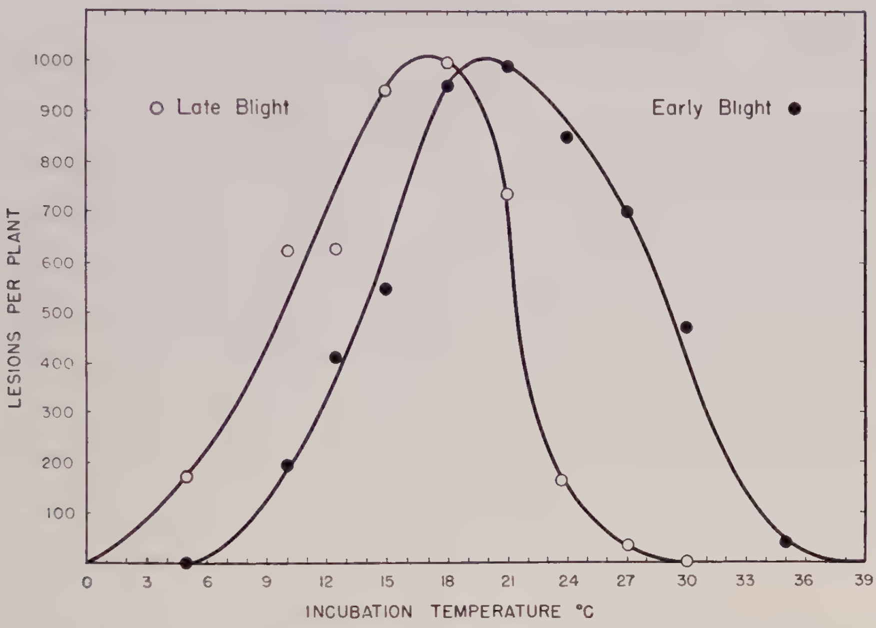

FIGURE 2. Effect of incubation temperature on infectivity of *Phytophthora infestans* (late blight) and *Alternaria solani* (early blight) on tomato plants (3 leaves).

Each chamber is lighted by 4 parallel 5-foot, 100-watt white fluorescent Mazda lights placed on the top of the chambers. Ordinarily the lights are used only when inserting or removing plants from the chambers. They are expected, however, to provide adequate light should it be necessary to leave the plants in the chambers for a more prolonged period than the customary 24 hours. The shelf across the center of each chamber is removable so that tall plants, such as potted apple trees, may be set on the bottom.

Hood. The hood constructed of Flexboard mounted on an alberene stone base measures 5 feet wide and 3 feet deep with a window centered in the front 36 inches wide and 30 inches high. Access to the hood can also be made through a side window 24 × 24 inches. The hood is operated by its own motor mounted above and at one side as illustrated in Figure 3 and the full capacity, at which it is generally operated, is 500 cubic feet of air per minute.

Turntable. The 18-inch compound turnable table, carrying 4 removable 4-inch secondary turntables, has been replaced by a stainless steel one of similar dimension shown in Figure 3. The turntable is motor-driven with motor and gear box mounted beneath the bottom of the hood. The shaft from the gear box is carried through the bottom of the hood and protected by a watertight sleeve. The main turntable is mounted 3 inches above the hood bottom and is geared for 15 r.p.m.

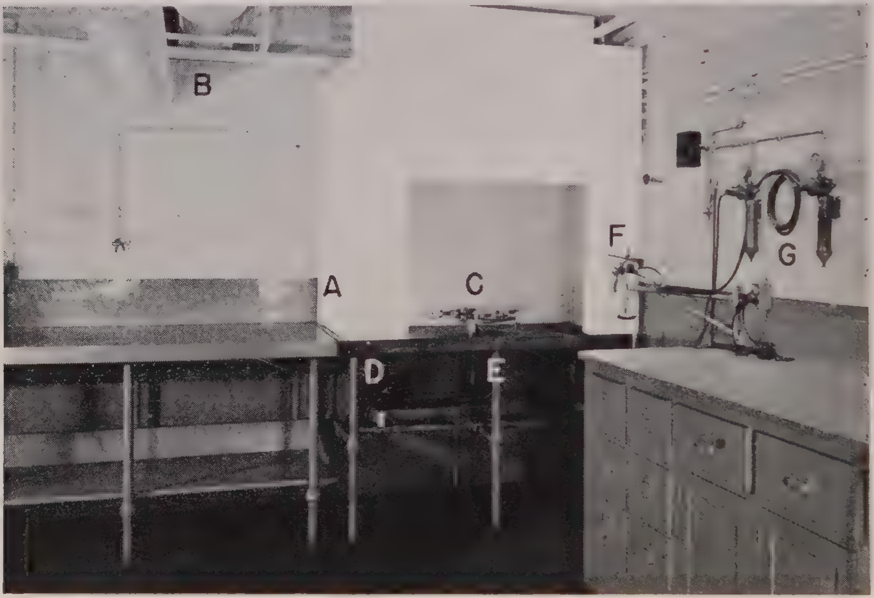

FIGURE 3. Hood, turntable, and spraying and inoculating assembly. A. Side window of hood, closed. B. Hood motor and draft control. C. Turntable with (D) motor and (E) gear box. F. Spray gun and stirrer swing to side position. G. Pressure gauges for spray gun and inoculation atomizer.

"*Rain.*" Plants are placed on the turntable to receive a "rain" treatment of tap water which is applied through a small rose type nozzle fastened to a ring stand about 2 feet above the turntable and 2 feet from the center. Or the "rain" may be applied by spraying tap or distilled water on the plants with the spray gun.

Spray gun and inoculation atomizer. This equipment, shown in Figure 3, is similar to that previously described and is similarly mounted and operated.

SUMMARY

A new basement laboratory for studying fungicides is described. The laboratory is equipped with insulated temperature-controlled incubation chambers, run at 100 per cent relative humidity and with fluorescent lighting. Internal chamber dimensions are 6×5×3 feet. One chamber is set at 70° F. for tomato early blight and another at 60° F. for tomato late blight and snapdragon rust as determined from incubation temperature-infection experiments. A stainless steel compound turntable for spraying, inoculating and "raining" on plants is motor driven at 15 r.p.m. and sets in a 5×3 foot hood with a capacity of 500 cubic feet per minute.

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CHARACTERISTIC CURVE FOR THE ACTION OF COPPER SULFATE ON THE GERMINATION OF SPORES OF SCLEROTINIA FRUCTICOLA AND ALTERNARIA OLERACEA[1]

S. E. A. McCallan

Workers concerned with the mode of action of chemicals on cellular organisms of all kinds have been much interested in the shape of the dosage-response curve, for by a study of the shape of this curve, possible hypotheses may be advanced to explain the mode of action. Unfortunately because of the variability of living organisms and the relatively small numbers that have been available in these studies, the resulting curves could be fitted by an embarrassingly large number of formulae, as has been pointed out by Clark (4).

Fungus spores present a rather unique opportunity for obtaining large numbers of organisms whose individual responses to the toxic action of chemicals may be determined readily. In this case the quantal response to be measured is germination. Accordingly a study has been made of the responses of a very large population of fungus spores to a soluble chemical toxicant, namely copper sulfate. Copper sulfate was chosen because of its general interest, its toxicity curve has already been studied to some extent, it is the basis of our most important group of fungicides and, being soluble, the errors of concentration and application are reduced to a minimum. It is hoped also that the information obtained, because of its extent, will provide some usable basic data for those interested in studying the form of dosage-response curves.

METHODS

The standard test tube dilution technique for use with the slide-germination method of evaluating protectant fungicides (1, 2) was employed with minor modifications. This technique consists essentially in preparing a series of dilutions of the chemical in test tubes, then adding a known quantity of fungus spore suspension and ultrafiltered orange juice spore germination "stimulant." Drops of the preparation are then pipetted onto glass slides held in moist chambers, and allowed to germinate.

Concentrations of chemical. Extreme care was taken to avoid errors of

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[1] Also presented before the American Phytopathological Society, Northeastern Division, Amherst, Mass., Nov. 26, 1946 (8). These studies have developed from some preliminary ones initiated by Dr. R. H. Wellman and the author.

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concentration when preparing the various dilutions of copper sulfate. Glass distilled water was used. The final concentrations of copper sulfate, allowing for dilution by spores and orange juice, ranged from 32.000 p.p.m. Cu in geometric steps of $\sqrt[4]{2}$, i.e. 1.189, down to 0.105 p.p.m. Cu. This gave a logarithmic series of 34 dosages.

Organisms. The two common test fungi studied were *Sclerotinia fructicola* (Wint.) Rehm. and *Alternaria oleracea* Milbraith (7). Both organisms were cultured on potato dextrose agar slants (9, footnote 9) at 21° C. The spores were obtained for use from 7-day-old cultures by the rubbing, centrifuging, and washing technique (10). The spore suspension concentration was adjusted to give a final concentration in the copper sulfate of 50,000 per cc. and the final orange juice concentration was 0.1 per cent.

Coding. In order to prevent any bias on the part of the operators when recording the germination results the identity of the individual test tubes of different concentrations was coded and the series arranged out of order.

Germination period. The spores were allowed a period of 48 hours to germinate in the presence of the chemical at 21° C. before germination counts were made. The extended period minimized possible further changes in germination during the time the counts were being taken. As has been shown previously (13), changes in per cent germination after 48 hours are very slight, and observations made during the period of counting showed no changes.

Replications. Paired drops from individual concentrations of copper sulfate were replicated ten times in five different moist chambers. As customary, 100 spores were counted from a set of paired drops. This gave ten independent counts of 100 spores each for each concentration, or a total of 1000 spores per concentration. In addition the entire test with each fungus was run three different times, several weeks apart. Because of the magnitude of the experiment only one fungus was tested at one time. In this manner there were six different tests run over a period of several months and 3000 spores were counted for each concentration and fungus. Thus with the number of dosages involved, observations on a total of approximately 100,000 individual spores were available for defining the dosage-response curve.

RECORDING RESULTS

Some preliminary work had shown that extreme care would be necessary in defining exactly the germination of a spore and in ascertaining not only the control germination but also prior germination.

Germination. A spore was defined as germinated if the germ tube protuberance exceeded half the minor diameter of the spore (1). At high concentrations of toxicant the identification of germination becomes of great importance for, as will be shown later, there is a high correlation between percentage germination and germ tube length. In the case of *Sclerotinia fructicola*, spores in chains and old dessicated misshapen spores were not included in the counts, while one-celled spores were omitted in *Alternaria oleracea*.

Prior germination. In both species it was observed that a very small percentage of the spores had germinated in culture or possibly after the water had been added but before the spores were placed in the copper sulfate, though the period of time elapsing in this latter case would not exceed 20 minutes. In each test counts were made of the spores germinating prior to setting up the test. The count was fairly uniform for each species and averaged 0.15 per cent for *Sclerotinia fructicola* and 0.31 per cent for *Alternaria oleracea*.

Control germination. The customary control spores were allowed to germinate in the absence of copper sulfate. Counts were made on 20 different lots of 100 spores each, that is, a total of 2000 spores for each test. The percentage germination was high in all cases and reasonably uniform for both species, averaging about 98 per cent for *S. fructicola* and 96 per cent for *A. oleracea*.

Observed non-germination. The results on the number of spores actually observed not to germinate out of a total of 100 spores counted are given in Table I for *Sclerotinia fructicola* and Table II for *Alternaria oleracea* for the various concentrations, replications, and tests. The ten entries for a given concentration and test represent the ten different replicate counts. There is no particular relation between the figures in any given vertical column as opposed to different columns. The 20 control replicate counts are also recorded, but as percentage germination. Observations on the four highest concentrations, not given above, appear in Table III in condensed form.

ADJUSTED PER CENT NON-GERMINATION

Because of the presence of prior germinated spores and non-viable spores in the controls, it is desirable to adjust the results on the basis of 100 per cent viable spores and no prior germination.

Let the percentage observed germination in the control $= C$, and in a given concentration or treatment $= T$ and let the prior germination $= P$.

The usual adjustment for combination of independent probabilities for adjusted percentage germination in treatment because of non-viable spores, i.e. non-germinated, in the control is:

$$100 \frac{T}{C} \tag{i}$$

TABLE I

Toxicity of Copper Sulfate to Spores of Sclerotinia Fructicola. Observed Number of Non-Germinated Spores Out of 100 Counted

Cu p.p.m.	Test 1										Test 2										Test 3									
0.105	4	2	1	1	0	4	1	2	4	2	4	0	1	3	1	1	0	2	1	3	2	2	2	4	3	2	3	1	3	1
0.125	0	0	2	2	1	2	0	2	2	2	3	2	5	3	1	2	5	0	2	1	0	2	6	1	1	3	2	4	5	4
0.149	2	1	0	3	4	2	2	2	2	4	4	1	3	2	2	1	4	0	0	3	2	4	3	5	4	4	5	4	1	4
0.177	2	1	2	2	0	1	2	3	1	3	1	2	3	3	0	1	2	1	2	1	5	5	7	5	4	4	3	3	6	2
0.210	2	4	1	1	2	4	2	2	1	4	3	1	1	3	4	3	1	2	1	1	5	6	5	4	3	3	3	6	6	6
0.250	3	7	3	5	4	2	8	4	4	2	1	5	4	1	5	2	1	1	2	3	3	6	6	4	3	5	6	8	9	4
0.297	4	2	3	2	3	3	6	5	3	4	2	2	4	4	7	3	2	3	1	1	3	5	6	1	4	3	6	4	3	4
0.354	7	2	1	2	5	2	3	4	3	2	3	2	3	4	1	1	2	2	2	4	5	6	5	4	3	5	5	7	6	3
0.420	3	5	2	2	1	2	2	4	2	4	2	4	6	1	1	2	2	2	5	8	2	3	9	9	8	5	4	3	6	7
0.500	5	4	3	3	1	1	2	4	2	4	4	9	3	1	3	1	3	1	1	3	3	4	12	5	6	8	5	5	10	7
0.594	6	7	7	3	9	1	3	4	2	4	10	7	5	2	1	1	3	4	6	2	17	7	13	14	10	8	11	9	5	6
0.707	3	8	5	9	3	5	3	4	4	5	8	4	7	5	10	4	2	4	3	4	9	6	9	11	12	11	10	10	6	5
0.840	9	7	5	6	7	9	5	10	4	3	5	2	3	3	7	4	3	2	5	7	7	12	21	9	14	11	9	10	6	8
1.000	18	22	18	30	49	38	17	25	12	16	4	4	12	5	25	12	6	5	8	8	30	27	30	33	37	28	13	14	17	18
1.189	26	33	48	38	49	39	38	29	41	34	11	9	12	21	30	24	3	9	3	9	53	45	34	31	24	32	68	36	35	41
1.414	48	60	38	49	44	44	65	52	40	47	12	15	22	19	26	12	10	12	14	10	56	63	37	41	34	26	53	62	40	49
1.682	76	70	77	66	75	29	61	45	62	64	26	17	48	59	51	51	24	40	41	34	80	76	73	70	52	55	86	76	80	84
2.000	81	79	71	84	71	66	51	70	68	76	23	20	52	36	68	64	20	36	68	64	94	88	64	71	77	65	87	92	89	90
2.378	85	77	81	90	83	82	79	85	95	91	70	70	74	77	69	66	61	69	68	71	93	92	69	68	84	69	89	95	93	88
2.828	81	75	66	76	89	83	82	83	86	82	65	73	69	64	67	75	68	66	71	69	88	77	91	97	95	94	96	97	94	92
3.363	76	77	87	89	93	85	92	93	86	88	66	59	81	79	68	65	70	68	79	82	98	97	99	99	98	C*	98	97	98	96
4.000	98	99	97	C	C	96	91	94	99	99	95	95	94	93	92	93	95	94	95	93	99	99	C	C	99	99	C	C	C	99
4.757	C	C	C	99	C	99	97	98	98	C	96	98	96	98	97	98	99	98	C	92	99	C	98	C	C	C	99	99	C	C
5.657	C	C	C	C	96	C	97	96	98	C	99	C	99	99	98	99	99	99	C	C	C	C	99	C	C	C	C	C	C	99
6.727	99	C	C	C	C	C	C	C	C	99	99	C	C	C	98	99	C	C	C	99	C	C	C	C	C	C	C	C	C	C
8.000	C	99	C	C	C	C	C	C	C	99	C	C	C	C	99	C	C	C	C	C	C	C	C	C	C	C	C	C	C	99
9.514	C	C	C	C	99	C	C	C	C	C	C	C	C	C	C	99	C	99	99	C	C	C	C	C	C	C	C	99	C	C
11.314	C	C	99	C	C	C	C	C	C	C	98	C	C	C	99	C	C	C	C	C	C	99	C	C	C	C	C	C	99	C
13.454	C	99	C	C	C	C	C	C	C	99	C	C	99	C	C	C	C	C	C	C	C	C	C	C	C	99	C	C	C	C
16.000	C	C	99	C	C	C	C	C	C	C	C	C	99	C	C	C	C	99	C	C	C	99	C	C	99	C	C	99	C	C
Control % germination	95	C	99	98	97	99	98	98	97	98	98	98	98	98	98	99	C	C	99	99	98	95	96	95	98	99	96	98	97	97
	99	C	96	98	96	98	C	98	C	C	94	97	C	98	97	98	99	98	98	C	96	94	98	96	96	96	98	99	98	96
Mean control	98.20										98.30										96.80									

* C = 100.

TABLE II

Toxicity of Copper Sulfate to Spores of Alternaria oleracea. Observed Number of Non-Germinated Spores Out of 100 Counted

Cu p.p.m.	Test 1										Test 2										Test 3									
0.105	3	4	6	5	2	5	5	2	7	4	3	4	5	4	3	7	3	4	3	4	2	6	4	2	2	2	2	0	3	2
0.125	6	6	6	3	6	4	2	4	5	5	3	6	9	5	5	5	4	4	4	7	2	5	1	4	4	1	6	2	3	2
0.149	5	3	2	4	3	1	4	3	3	5	7	4	6	7	5	3	3	9	6	4	1	5	4	6	1	2	4	1	4	0
0.177	6	3	7	4	6	6	4	3	4	3	7	3	4	5	7	4	8	6	9	7	2	4	6	5	3	2	4	3	2	5
0.210	4	4	6	4	7	6	3	3	3	6	5	6	12	4	4	2	3	8	3	7	4	2	7	2	5	2	4	4	3	4
0.250	4	3	5	6	3	4	5	4	5	5	6	6	4	8	3	5	6	4	6	6	9	3	7	3	6	8	2	4	5	6
0.297	4	6	6	5	5	2	3	5	3	4	8	6	10	8	7	6	5	8	5	2	5	4	2	3	4	1	5	3	6	2
0.354	8	3	5	3	8	4	3	6	6	4	5	7	5	9	7	4	4	7	6	5	4	5	8	7	7	5	3	5	6	9
0.420	3	6	4	8	6	5	6	5	7	5	10	6	6	10	8	5	6	9	4	1	6	10	3	5	4	8	5	3	7	3
0.500	8	6	12	7	7	6	4	7	8	8	6	3	9	13	8	7	9	5	5	5	5	6	6	4	7	3	7	9	1	5
0.594	7	7	4	2	7	5	8	4	10	5	7	9	10	10	7	7	11	4	6	5	9	9	9	3	7	6	8	5	3	3
0.707	3	6	9	12	9	8	5	8	5	9	7	9	13	12	12	9	13	9	7	6	12	7	12	8	14	11	19	18	10	12
0.840	5	8	5	8	12	9	10	18	8	6	13	6	23	14	9	10	11	9	10	11	8	9	13	6	13	14	19	15	8	11
1.000	7	5	15	8	9	6	4	6	13	6	12	12	11	15	16	13	10	19	16	17	16	15	11	18	18	27	17	15	15	21
1.189	16	8	14	13	12	14	9	14	12	21	10	16	28	11	13	12	32	54	26	19	6	17	14	24	29	14	14	11	13	8
1.414	13	8	17	14	15	9	6	11	9	8	21	12	29	34	21	16	67	66	70	41	12	7	18	23	18	21	14	14	44	55
1.682	8	17	16	11	14	10	22	15	12	10	26	24	63	32	34	53	73	67	58	21	18	29	44	71	61	67	25	18	68	52
2.000	17	23	16	14	15	13	21	27	13	18	26	30	32	20	34	24	27	16	52	31	27	28	31	27	51	36	41	26	36	28
2.378	19	23	15	20	18	27	30	27	21	29	58	51	77	71	50	82	77	80	83	86	49	36	59	63	87	82	55	54	84	76
2.828	37	38	46	63	41	40	55	53	43	46	91	84	78	70	38	58	50	62	53	84	87	74	73	69	95	86	84	68	86	85
3.363	59	39	51	51	74	59	43	37	86	82	63	90	92	97	95	94	93	94	94	95	98	99	92	90	94	96	94	94	98	99
4.000	56	60	69	57	93	91	68	67	90	91	90	80	96	95	86	94	86	94	89	89	94	92	93	90	96	97	95	89	95	94
4.757	94	89	61	66	98	96	98	94	93	93	85	85	92	96	94	96	89	92	90	92	98	95	73	83	98	C*	96	99	C	97
5.657	98	99	97	91	99	97	97	98	99	C	91	93	93	93	97	99	98	97	98	97	C	C	99	98	99	98	99	C	C	99
6.727	98	C	97	98	C	97	98	C	C	C	C	99	C	99	99	98	C	C	C	C	C	99	C	C	C	C	C	C	C	99
8.000	C	99	99	C	99	98	99	99	C	99	C	C	C	C	C	C	C	C	99	C	99	C	C	99	C	C	C	C	C	99
9.514	99	C	C	98	C	C	C	C	C	99	99	99	98	C	C	C	C	99	C	99	99	C	C	C	C	C	99	99	C	C
11.314	C	99	99	C	C	99	C	C	99	C	C	C	99	C	C	C	C	C	C	C	C	C	C	99	C	C	C	C	C	C
13.454	C	C	C	C	C	C	C	C	C	99	C	99	C	C	C	99	99	99	99	99	C	C	C	C	C	C	C	C	C	C
16.000	C	C	99	C	C	C	C	99	C	C	C	99	C	99	C	C	C	C	C	C	C	99	C	C	C	C	C	C	C	C
Control % germination	92	96	99	98	99	95	97	96	97	92	97	95	96	93	95	96	97	97	92	96	99	99	97	97	95	97	99	96	95	97
	95	95	93	92	98	99	96	98	95	96	96	98	95	97	95	91	96	95	94	96	99	98	95	98	96	99	98	99	95	97
Mean control	95.90										95.35										97.25									

* C = 100.

TABLE III

Number of Observations in Which 99 Spores Non-Germinated Out of 100 Counted. Remaining Observations, from Total of 10, All 100 Spores Non-Germinated

Concn. p.p.m. Cu	*Sclerotinia fructicola*			*Alternaria oleracea*		
	Test			Test		
	1	2	3	1	2	3
19.027	1	2	0	1	1	0
22.627	1	0	2	2	1	0
26.909	3	0	1*	1	0	0
32.000	0	2	0	0	0	1

* 1 observation of 98 spores non-germinated.

However, the control spores should be adjusted first for prior germination, thus

$$100\,\frac{C - P}{100 - P} \tag{ii}$$

It is also necessary to adjust treated spores for prior germination, thus

$$100\,\frac{T - P}{100 - P} \tag{iii}$$

Then by combining the two adjustments, (ii) and (iii), to give a corrected percentage for non-viable spores in the treatment as in (i), we have

$$100\,\frac{100\,\dfrac{T - P}{100 - P}}{100\,\dfrac{C - P}{100 - P}} \qquad \text{or } 100\,\frac{T - P}{C - P} \tag{iv}$$

This expressed as adjusted non-germinated spores is

$$100 - 100\,\frac{T - P}{C - P} \qquad \text{or } 100\,\frac{C - T}{C - P} \tag{v}$$

Example: 0.2 per cent spores prior germinated, 97 per cent observed germination in control, 80 per cent observed germination in treatment. Then adjusted non-germination in treatment from formula (v) is:

$$100\,\frac{97 - 80}{97 - 0.2} = 17.56$$

All data were accordingly adjusted in this manner for each test. In order to obtain the data in more compact form for further study, it seemed advisable to combine the concentration data in pairs, thus 0.105+0.125 p.p.m. Cu, and 0.149+0.177 etc., which, since the series was logarithmic, have been averaged logarithmically. The percentages non-germination from the six tests, i.e. three tests for each of two concentrations, were averaged arithmetically and the data are summarized in Table IV for *S. fructicola* and *A. oleracea*. The data for convenience are also expressed as log concentration and probit non-germination. It is to be noted that each non-germination percentage recorded is the result of individual observations on 6000 spores. The results at the highest concentrations have been omitted in this table because the very small number of spores germinating and the difficulties of accurately defining germination introduce relatively large errors. However, it would be possible to interpret the data, that there is no further change at this level in percentage germination, that is, 100 per cent non-germination may have been attained.

THE DOSAGE-RESPONSE CURVE

Probit-log dosage. From the work of Gaddum (5) and Bliss (3) it has been found that for many kinds of organisms the logarithms of the individual dosages appear to be normally distributed. This distribution will give the now generally familiar straight line relation when the percentage response expressed as probits (3) is plotted against the logarithm of the dosage, or, as is commonly done (9), arithmic values may be plotted on logarithmic probability graph paper. However, when the characteristic curve (4) for copper sulfate and the above fungus species are plotted in this manner from the data of Table IV, it will be seen in Figure 1 that a

TABLE IV

Combined Adjusted Dosage-Response Results

Concentration p.p.m. Cu		Per cent non-germination of spores			
		Sclerotinia fructicola		*Alternaria oleracea*	
Mean	Log	Mean	Probit	Mean	Probit
0.115	0.0593	0.052	1.728	0.440	2.380
0.162	0.2098	0.569	2.469	0.922	2.643
0.229	0.3603	1.512	2.833	1.318	2.779
0.324	0.5108	1.443	2.815	1.718	2.884
0.458	0.6613	1.996	2.946	2.741	3.080
0.648	0.8119	4.315	3.285	4.829	3.338
0.917	0.9624	11.431	3.796	8.688	3.640
1.297	1.1129	32.115	4.535	17.444	4.063
1.834	1.2634	61.612	5.295	28.168	4.422
2.594	1.4139	79.477	5.823	57.391	5.186
3.668	1.5644	91.160	6.351	83.260	5.964
5.187	1.7150	99.048	7.345	94.329	6.583
7.336	1.8655	99.981	8.549	99.785	7.855
10.375	2.0160			99.994	8.830

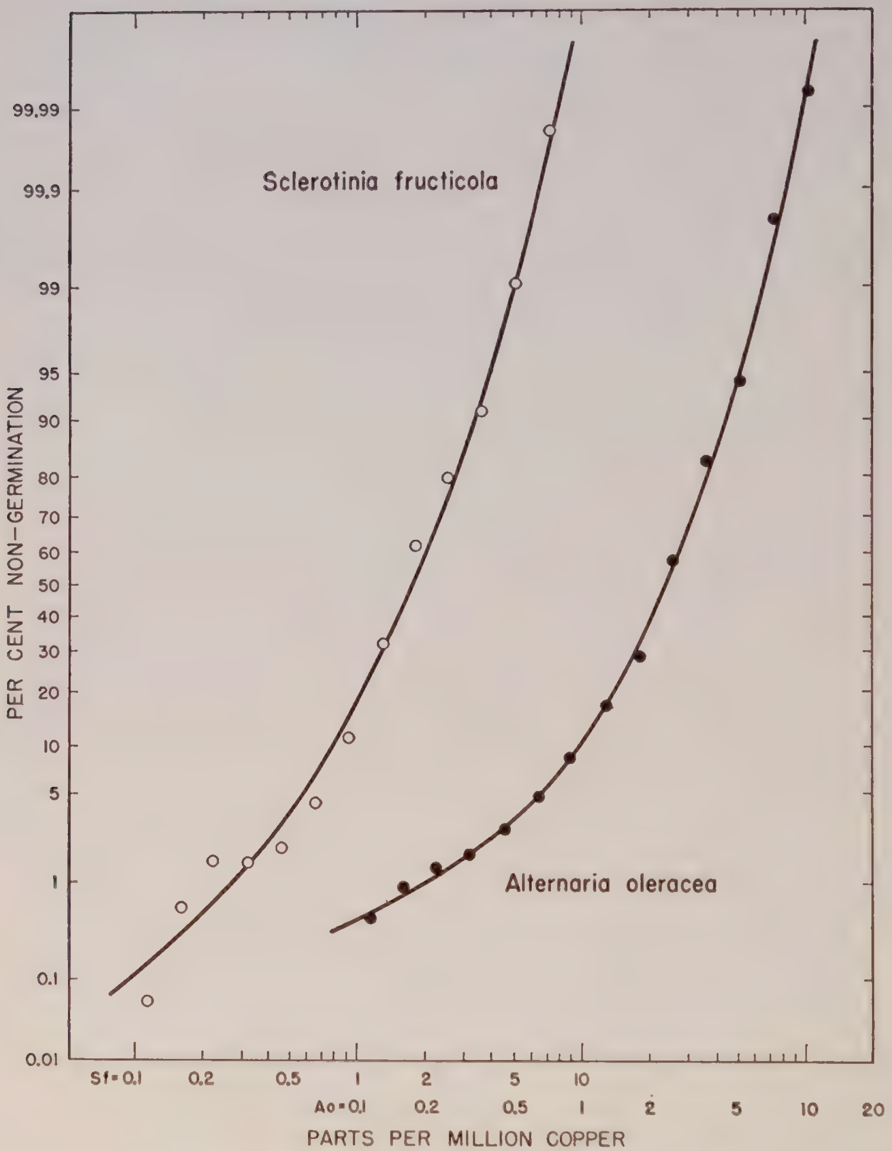

FIGURE 1. Dosage-response curves for action of copper sulfate on germination of spores of *Sclerotinia fructicola* and *Alternaria oleracea*, plotted on logarithmic probability scale. Note overlapping concentration scale, 1 p.p.m. for *S. fructicola* coincides with 0.1 p.p.m. for *A. oleracea*.

straight line definitely does not result. Nor can it be interpreted as a main straight line which "breaks" at the lower dosages giving a secondary straight line as has been shown by Bliss (3) and others. Instead there is a definite smooth curve. Evidently the response of these spores to copper sulfate cannot be explained by this theory. This confirms previous but much less extensive data (9) in which it was found that these curves were concave when probit response was plotted against logarithm of dosage.

Adsorption equation. The fixation of poisons by cells appears in many cases to be due to adsorption (4) and the Langmuir adsorption equation commonly has been applied to such data with more or less success. This equation in the form $kx^2 = y/100 - y$ (3) when applied to the above fungus spore data, however, would not give a satisfactory fit over the entire length of the curve.

THE α EXPONENTIAL TRANSFORMATION

In developing his Theory of Variability to explain the mechanism of fungicidal action, Parker-Rhodes (11) recognized that in many cases plotting probit kills, i.e. non-germination, against log concentration did not give straight line responses. He proposed instead an exponential transformation of x which plotted against probit y would give a straight line. The exponential power is called α or the Index of Variation. Thus the spores are said to be normally distributed against the concentration x raised to some power α. "α can . . . be estimated by plotting x^α against y for various values of α, and choosing that value giving the best straight line" (11, p. 127). Incidentally, α in turn is used to derive the basic concept of this theory namely, Variability or U; $U = 1/\alpha^2 m^2 b^2$.

The copper sulfate data of Table IV were subjected to the exponential transformation and it was found that the best values for α were 0.41 and 0.56 respectively for *Sclerotinia fructicola* and *Alternaria oleracea*. The best value of α is that which gives the smallest value for the errors of estimate, $Sy^2 - (Sxy)^2/Sx^2$. The regression equation for probits on concentration$^\alpha$ for *Sclerotinia fructicola* was $\hat{Y} = 1.354X + 0.590$ and the standard error of estimate, $s_{y \cdot x} = 0.207$. The standard error of the regression coefficient s_b was 0.039 which gives a highly significant t value of 34.705 (12). Likewise, the regression equation for *Alternaria oleracea* was $\hat{Y} = 0.525X + 1.873$, $s_{y \cdot x} = 0.297$, $s_b = 0.021$, and $t = 24.897$ which is highly significant. A comparison of the regression coefficients for each fungus gave a t value of 18.702 (6, p. 42) thus showing a highly significant difference. The data are also plotted in Figure 2 where the excellent fit may be observed as well as the difference in slope.

It is evident that the more versatile exponential transformation to straighten dosage-response curves is an advantage over the commonly used logarithmic transformation and appears equally logical. However, it

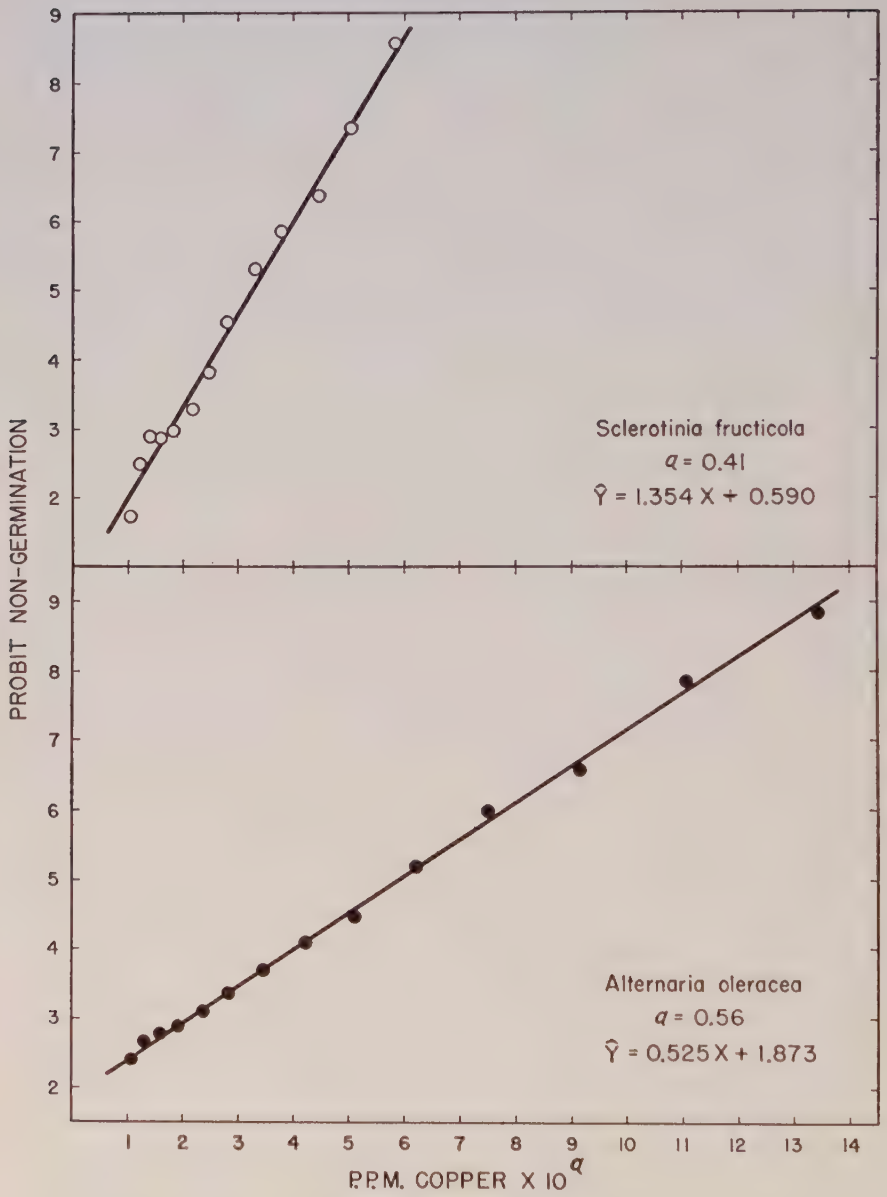

FIGURE 2. Straight line dosage-response curve for action of copper sulfate on spore germination of *Sclerotinia fructicola* and *Alternaria oleracea*. Probit non-germination plotted against α exponential transformation of concentration.

is to be noted that this transformation as employed by Parker-Rhodes can be used only on the concave or J type curves. For the less common convex curves which "break" and flatten at the top such as that of the action of zinc chloride on fungus spores (9) it will be necessary to use a negative exponential transformation.

RELATION BETWEEN GERM TUBE LENGTH AND PERCENTAGE GERMINATION

It is a common practice among investigators observing the toxic action of fungicide on germinating spores to record not only the percentage germination but also an estimate of the germ tube length. In fact a few

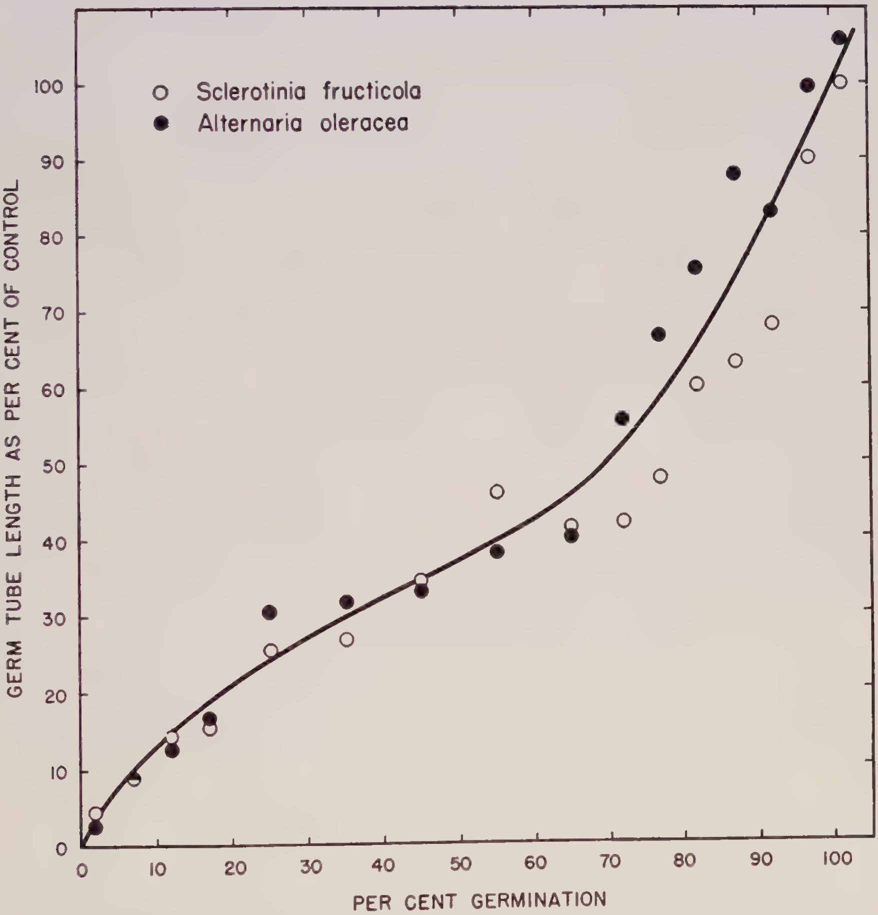

FIGURE 3. Relation between germ tube length and percentage germination for spores of *Sclerotinia fructicola* and *Alternaria oleracea* in copper sulfate solution.

TABLE V

FREQUENCY DISTRIBUTION OF GERM TUBE LENGTHS ON PERCENTAGE GERMINATION

Germ tube length as % of control	Per cent germination >0 4	5 9	10 14	15 19	20 29	30 39	40 49	50 59	60 69	70 74	75 79	80 84	85 89	90 94	95 99	100
A. *Sclerotinia fructicola* in copper sulfate																
150–159															1	
140–149																
130–139															14	9
120–129															1	
110–119															12	20
100–109													1	1	62	37
90–99															1	1
80–89								1			1	2	1	5	64	31
70–79													1	7	36	8
60–69								1	1		1		4	11	24	3
50–59					2		2	6	4	3	2	4	7	6	13	
40–49					1		1		5	2	2	2		1	1	
30–39					4	12	1	3	10	4	5		1	1		
20–29			2	4	9	22	6	2	2	1						
10–19	6	9	8	9	11	5		1								
5–9	22	11	1	3												
> 0–4	58	5	2	1												
Total = 667	86	25	13	17	27	39	10	14	22	10	11	8	15	32	229	109
B. *Alternaria oleracea* in copper sulfate																
150–159													2	1	10	10
140–149														1	4	3
130–139																
120–129													4	5	33	15
110–119												1		1	16	7
100–109												2	10	4	16	6
90–99										1	1	4	5	15	54	22
80–89												1	1	1	8	4
70–79								1		1	9	5	12	16	39	10
60–69					2	1	3	1	2	5	1		5	10	12	5
50–59					1	3	2		1	1	3	4	2	5	5	1
40–49					1		2	1	3	6	3	2	1	5	3	
30–39		1	1	2	1	4	2	6	2			1	2		2	
20–29		3	3		1	1	6	4	3							
10–19	1	7	4	6	4	5	5		1	1						
5–9	6	13	5	3	2	1	1									
>0–4	80	12	4													
Total = 675	87	36	17	11	12	15	21	13	12	15	17	20	44	64	208	83

investigators have preferred the latter to the former. While it seems more or less agreed that there is a correlation here, no quantitative studies appear to have been made.

In the process of obtaining the germination results for the copper sulfate data above, estimates were also made of the "average" germ tube length for each population of 100 spores. That is, for each entry in Tables I and II there was a single estimate, based on an ocular micrometer, for the average or typical germ tube length for the 100 spores in that particular environment. It is of course impractical to attempt individual measurements of germ tubes by an ocular micrometer as few germ tubes even approach straightness.

The results were then expressed as a percentage of the control germ tube length and the data grouped by adjusted per cent germination and germ tube length classes and summarized in a frequency distribution table (Table V). For example, for *Sclerotinia fruticola* within the group 95 to 99 per cent germinated, 14 observations showed germ tube lengths from 130 to 139 per cent of the control. These results have also been plotted and are shown in Figure 3 as an asymmetrical sigmoid curve. It will be observed that this curve is the inverse of the usual arithmetic plot dosage-response curve, in that the former approaches the y axis in an asymptotic manner rather than the x axis. No simple and logical function has been found to express the relation of germ tube length to percentage germination, though it is evident that they are highly correlated and that germ tube length increases with increasing per cent germination.

SUMMARY

Extensive data were obtained for the dosage-response curve of copper sulfate on spore germination inhibition of *Sclerotinia fructicola* and *Alternaria oleracea.* Thirty-four dosages from 0.105 to 32.000 p.p.m. Cu with a dosage ratio of $\sqrt[4]{2}$ were used, 1000 spores counted at each dosage and the entire test run three times, thus giving about 100,000 spores for determining the characteristic curve.

Elaborate precautions are necessary for defining germination and ascertaining prior germination and non-germination of the control spores. The final per cent non-germination for treated spores is derived from the formula $100\,\frac{C-T}{C-P}$, where C and T are percentages of observed germination in control and treated spores respectively and P is the percentage of prior germination in the control.

Smooth curves concave upwards are obtained when probit non-germination is plotted against logarithm of concentration. The Langmuir adsorption equation also fails to give a satisfactory fit.

By means of the Parker-Rhodes α exponential transformation of concentration a straight line will result for the regression of probit non-germination on concentration$^{\alpha}$. For *S. fructicola* $\alpha=0.41$ and $\hat{Y}=1.354X+0.590$ and for *A. oleracea* $\alpha=0.56$ and $\hat{Y}=0.525X+1.873$. These regressions are highly significant.

It is necessary, however, to use a negative α transformation to straighten convex curves of the zinc chloride type.

The positive or negative exponential transformation of concentration appears likely to give more satisfactory results with spore germination responses than the logarithmic transformation and seems equally logical.

Germ tube length expressed as per cent of control when plotted against percentage germination gives an asymmetrical sigmoid curve with germ tube length increasing with increasing per cent germination.

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EVALUATION OF CHEMICALS AS SEED PROTECTANTS BY GREENHOUSE TESTS WITH PEAS AND OTHER SEEDS[1]

S. E. A. McCallan

The interest in evaluating new chemicals as possible fungicides emphasizes the necessity of suitable methods for discovering such potential materials. The problem here is not with a few preparations reported of interest by others, but rather with the hundreds or more of untried chemicals which may be of concern to certain workers in the initial stages of investigations. Thus there is need for simple rapid screening methods to reveal the few promising materials deserving of the more elaborate field and practical tests. However, because of the specificity of organic as well as inorganic compounds it is necessary to have methods representing at least the more important types of diseases (9). Among the major types are the so-called damping off diseases of seeds and seedlings.

Numerous studies have been made on the damping off diseases especially with a view to practical control by the use of seed protectants and reference may be made to the work of Horsfall (7), Kadow and Anderson (8), and Taylor and Rupert (14) both for general background and literature. The work of Horsfall and his associates has shown that among various seeds, peas would be particularly suitable for use in a test method. Accordingly quantitative studies have been made on various factors deemed of importance in developing a pea seed method suitable for evaluating chemicals as possible seed protectant fungicides. These studies have also been extended in a limited way to various other seeds. It is believed that a simple, practical, and satisfactory method has resulted from these studies performed over a three-year period.

METHODS

Certain of the procedures to be described here were adopted because of satisfactory common usage or practical considerations.

Seed. Pea seed (*Pisum sativum* L.) of the variety Perfection was selected for use at the suggestion of Dr. J. G. Horsfall. This is a wrinkled pea and unusually sensitive to soil organisms.[2] As will be shown later, untreated

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[1] A preliminary report was presented before the American Phytopathological Society, Cincinnati, Ohio, December, 1946 (10).

[2] The seed has been kindly furnished by Dr. Floyd L. Winter, Associated Seed Growers, Inc., 205 Church Street, New Haven, Connecticut.

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seeds in sterile soil gave a high percentage of germination. The stock seed was stored in a tight container held in a dry room at 5° C. to maintain its viability. A few comparative tests have been made with some other vegetable seeds. These will be discussed later.

Chemicals. Since dust treatments of seed are so much more practical than wet ones no attempt has been made to modify the method for wet treatments. Liquid chemicals, however, may be incorporated on dust diluents for dry seed treatments. In addition to the numerous experimental chemicals, four common commercial seed protectants have been used extensively in these studies particularly as standards. These are Semesan[3] (30 per cent hydroxymercurichlorophenol), Arasan[3] (50 per cent tetramethylthiuram disulfide), Yellow Cuprocide[4] (97 per cent cuprous oxide) and Spergon[5] (98 per cent tetrachloroquinone). In order to bring out various relations these standard fungicides were used frequently at dosages much lower than generally recommended.

Treatment of seed. In most cases seeds were treated in units of 100. This amount of Perfection pea seed weighs approximately 20 grams. If more seed is required for planting or if the dosage is very small, larger units of peas would be used. Similarly for very small seeds of other vegetables, more seeds but less weight would be used. However, in all cases the seeds were placed in extra wide-mouth specimen bottles of 1, 2, 4 or 8-oz. capacity. The size chosen was such that the seeds approximately half filled the bottle. The appropriate size for 100 peas is 2-oz. Most commercial seed protectant dusts are recommendated at a dosage at or near 0.25 per cent of seed weight. Accordingly dosage series have been standardized to include this level and to vary four-fold, thus a complete series would be 4.0, 1.0, 0.25, 0.0625, and 0.0156 per cent of seed weight. The higher dosages have been used to demonstrate seed injury, if any, and the lower dosages to detect chemicals of unusual fungicidal activity. The required amount of chemical dust is weighed on an analytical balance; the minimum quantity weighed is 50 milligrams. The chemical is added to the seed contained in the specimen bottle which is corked. The seeds are well coated with the chemical by rolling horizontally for one-half hour on a rolling machine illustrated in Figure 1. Two-oz. bottles will revolve about 200 times per minute.

Soil. Earlier attempts to infest soil with pure cultures of various organisms gave erratic responses in different flats and were discontinued in favor of naturally infested soil. The use of the naturally infested soil is considered more practical, simpler, and highly satisfactory. In any case soil artificially infested with pure cultures will not necessarily maintain that balance. Other advantages of naturally contaminated soil have been discussed by Horsfall (7). A sample of naturally infested soil was obtained from Bethany, Connecticut through the kindness of Dr. J. B. Skaptason.[6] As will be explained in more detail later, the soil before use is diluted by thoroughly mixing with fresh greenhouse soil. The greenhouse soil is a Gloucester loam sod composted with cow manure, ratio of 4 to 1, for at least three years. After use the infested soil is composted with the pea seedlings in large covered boxes, held indoors, for several months and is then ready for use again following further dilution with fresh greenhouse soil. The soil has been handled in this manner for three years and has continued to maintain a high level of inoculum potential, with no evidence of accumulation of toxic chemicals in the soil, and with the untreated checks

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[3] duPont Semesan Co., Inc., Wilmington 98, Delaware.

[4] Röhm and Haas Co., Inc., Philadelphia, Pennsylvania.

[5] United States Rubber Company, Naugatuck Chemical Division, New York, New York.

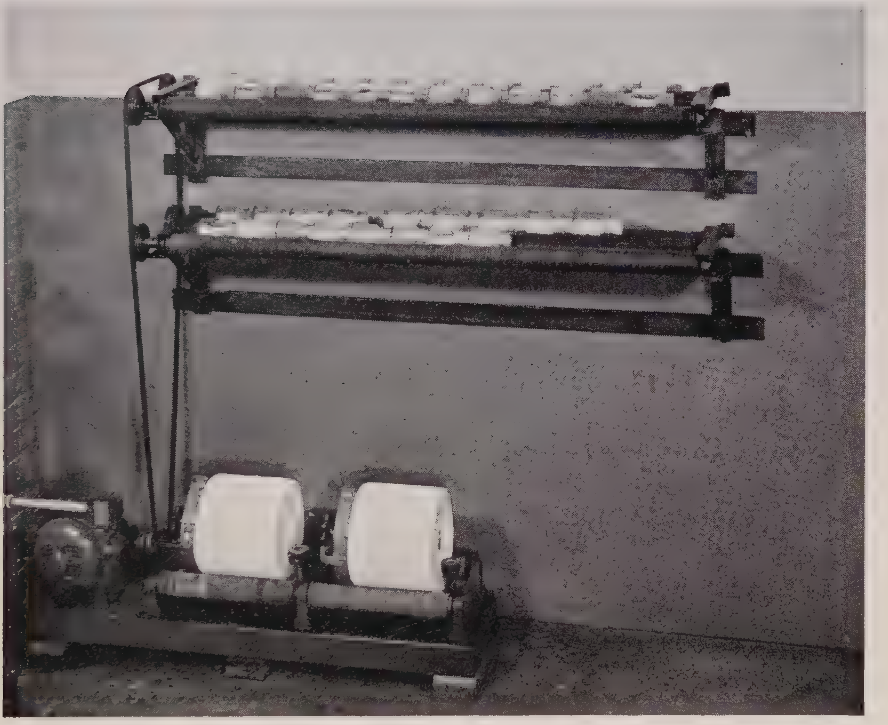

Figure 1. Rolling machine for coating seeds with chemical dust. Spacing of rollers adjustable. Pea seeds in 2-oz. bottles, except lower background of 4-oz. bottles. Ball mill at base driven by same motor.

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[6] Formerly of Naugatuck Chemical Division of United States Rubber Company, Naugatuck, Connecticut.

showing no consistent change with time. The pH of the soil averages about 6.0. Limited isolations indicate that the dominant organism is a *Pythium*.

Planting. The soil is placed in small flats (10×13 in., inside) or large flats (13×18-1/2 in., inside) or more rarely directly in greenhouse benches. The small flats are preferred and five or six shallow furrows are pressed out with a beveled edge crosswise of the flat. Ten pea seeds are removed individually from the bottle with forceps and placed in each furrow. Each row is labeled and soil added to the required depth, lightly pressed down and the flat immediately watered. With the large flats the rows are run lengthwise and divided in two, so that in all cases the basic unit is 10 pea seeds. The flats are held in a greenhouse run at about 70° F. night temperature and kept well watered.

Controls. Controls are of two kinds, seed and soil. Seed controls consist of a single flat of steam sterilized soil in which untreated seed is planted. These controls on seed viability are usually carried with each test or at least at frequent intervals and so far have always shown a consistently high viability of from 88 to 98 per cent, averaging 94 per cent seedlings. For very precise comparison of results and also where the seed control percentage germination is not high, the usual correction for the control germination will be necessary (1). Soil controls consist of untreated seed planted in infested soil, to check on its inoculum potential. They necessarily are carried for every test and usually consist of rows randomized among the various flats of treated seed or they may constitute one or more entire control flats. However, in general there are no more units of controls than of any one treatment. This efficient arrangement will be elaborated later. The soil controls range from about 3 to 30 per cent emergence of seedlings with an average of 12 per cent. Typical examples of seed control, soil control, and treated seed in infested soil, 14 days after planting, are shown in Figure 2.

Observations. Observations may be taken on the number of seeds that have emerged up to any given time, i.e. Emergence, and on the number of seedlings that are standing at any given time, i.e. Stand. Also height may be recorded. The difference between emergence of a treatment and of the control has been called by some pre-emergence damping off, while the difference between emergence and stand is known as post-emergence damping off. These terms are debatable and may be confusing. For example, seedlings that fail to appear supposedly because of pre-emergence damping off also may be failing because of lack of viability, seed decay, and injury from the chemical. Evidence will be given later that under these conditions seed treatments do not control post-emergence damping off. Hence when dealing with experimental seed treatments the expression damping off is to be avoided in recording results and the direct and measurable emergence and stand are to be preferred.

RELATIONS BETWEEN EMERGENCE, STAND, HEIGHT AND TIME

It is desirable to know the relation between emergence, stand, seedling height, and time, and which criterion is the most suitable for recording results.

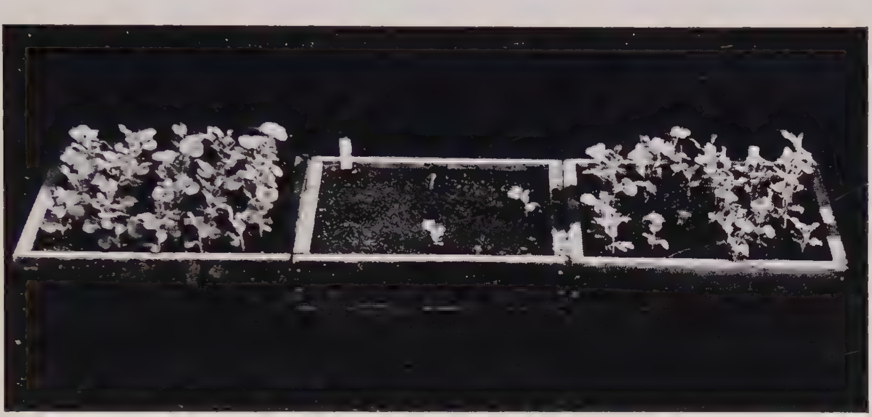

FIGURE 2. Typical examples of seedling emergence at 14 days. Left, untreated seed in sterile soil; center, untreated seed in infested soil; right, experimentally treated seed in infested soil. Ten seeds planted in each row, 6 rows per flat.

TIME

The effect of time on emergence, stand, and height was studied for a series of chemical treatments of varying efficacy. Observations were taken 3, 5, 7, 10, 15, 20, 25 and 30 days after planting. The summarized results of several tests at different times on a total of 4360 seeds are shown graphically in Figure 3. It will be seen that the seedlings begin to emerge at the third or fourth day and that emergence is complete by the 15th day. Stand, on the other hand, does not begin to be appreciably affected until most of the seedlings have emerged. The diminishing stand due to post-emergence damping off continues with further time, though at a lessening rate by the 30th day. Seedling height increases rapidly but toward the end of this period begins to fall off due to crowding in the flats. The appearance of seedlings treated with a series of the same fungicides are shown in Figure 4 at 7, 10, 14 and 21 days after planting.

REGRESSION OF HEIGHT ON STAND

The relation between height and percentage stand has been determined for two typical tests on units of 50 or 60 seeds per treatment and the significance of the regression calculated (13). The data are summarized in Table I and a graphical presentation for one of the tests is shown in Figure 5. It will be noted that, while the regression is not significant at 7 days in

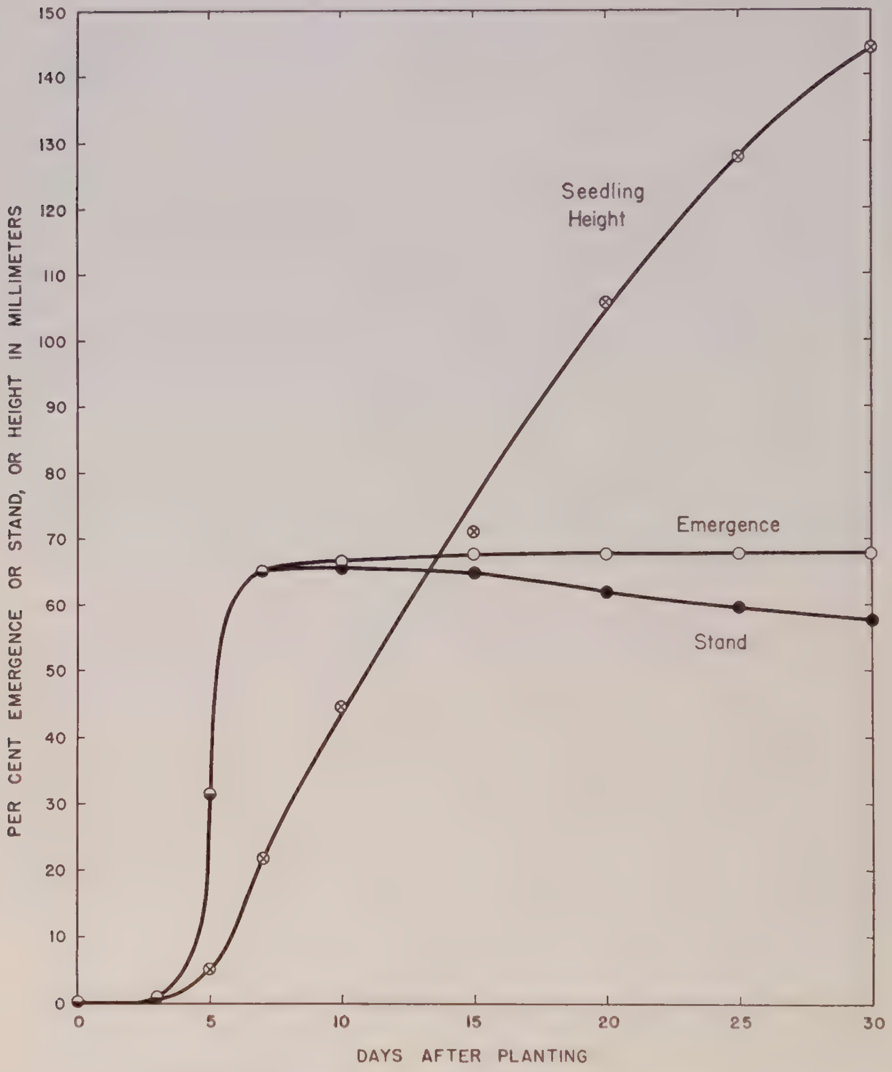

FIGURE 3. Typical observations of emergence, stand, and height of pea seedlings at different times.

one test and at 30 days for the second test, throughout the more important middle range the significance is marked. In general there is thus little to be gained in the laborious recording of seedling height since it is so closely dependent on stand.

EMERGENCE AND STAND

It is a general opinion that a good seed protectant will control both pre and post phases of damping off, that is, it will improve not only emergence

TABLE I

SIGNIFICANCE OF REGRESSION OF HEIGHT OF SEEDLINGS ON PER CENT STAND AT DIFFERENT AGES

Age in days	*t* Value	
	Test 250	Test 254
7	3.68**	1.34
10	4.98**	3.59**
14	13.98**	15.48**
20	2.72*	2.65*
30	1.87	3.24**
Degrees of freedom	29	48

* Significant; odds of 19:1 to 99:1.
** Highly significant; odds greater than 99:1.

but also stand. Seemingly it is also possible for some treatments to be effective in one type of control and not in the other. This relation between percentage emergence and percentage stand has been studied for a large number of exploratory compounds as well as the four standard materials listed above. The data were accumulated in some 10 different tests run over a period of time. By the method of covariance (13) it is possible to adjust the percentage emergence to a common level (within a test) and then test for the significance of differences between the adjusted stands. With the emergence data on an equal basis any differences in stand must be due to control of post-emergence damping off. That is, the treatments would differ in their ability to improve stand, i.e. control post-emergence damping off as distinct from their efficiency in improving emergence.

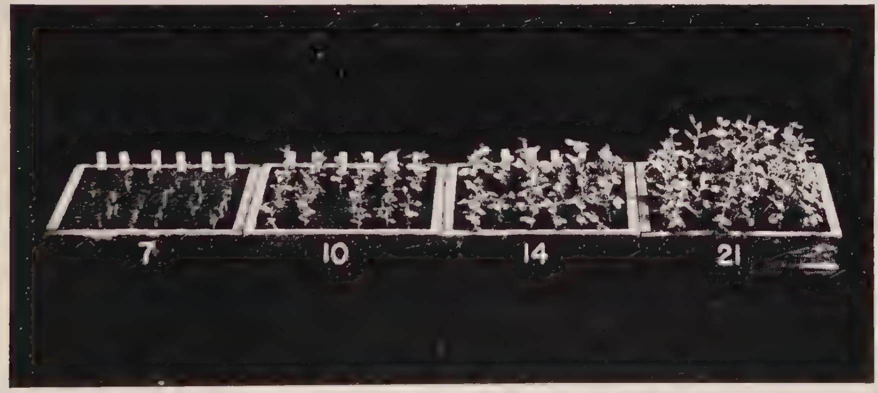

FIGURE 4. Appearance of pea seedlings at different days after planting from seeds treated with a series of the same fungicides.

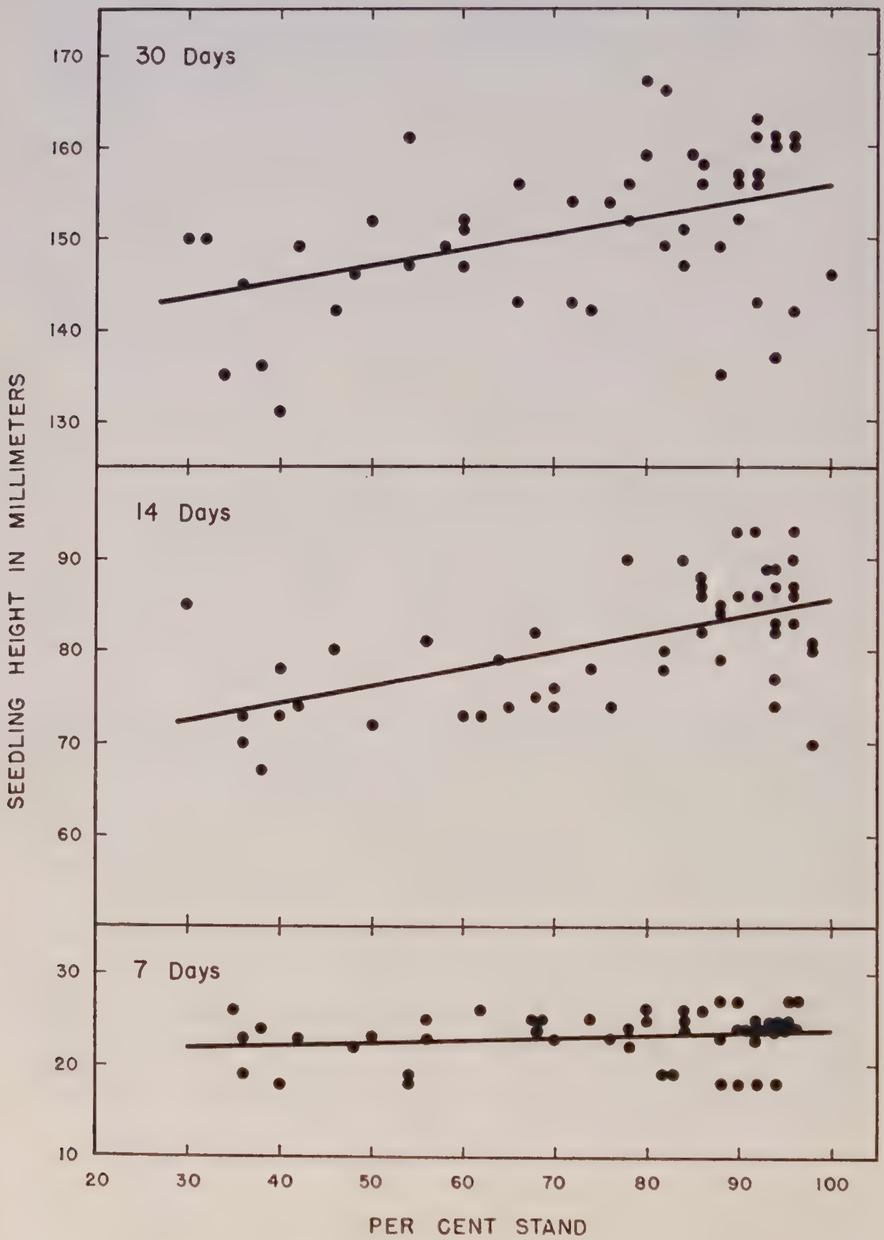

FIGURE 5. Regression of pea seedling height on per cent stand at different times. Data of Table I, Test 254, 50 seeds per observation.

The summarized data for the tests of covariance are given in Table II, where it will be seen that in only two cases out of 10 did the stands differ significantly. This first block of data was calculated on results taken at 30 days. For comparison two tests, one non-significant, No. 250, and the other significant, No. 261, were also calculated at the 14-day level and no appreciable change was found. The angular transformation under some circumstances is considered more appropriate for analysis of percentage data (13, p. 447) with demonstrable significance for smaller differences. Accordingly the data of test No. 250 were converted to equivalent angles but led to practically identical results as before, so it was not considered profitable to continue this transformation. An examination of the two tests showing significant differences in stands did not reveal any unusually effective treatments not appearing in other tests.

TABLE II

Non-Significance of Differences in Treatment Stands When Adjusted to Common Emergence Level by Covariance

Test No.	Treatments		Error		F Value
	D.F.	M.S.	D.F.	M.S.	
250	21	3.19	109	2.05	1.55
254	39	1.75	159	1.42	1.23
256	23	1.14	119	1.05	1.08
261	23	2.31	95	0.83	2.78**
285	5	0.90	35	1.02	0.88
289	9	2.65	39	0.28	9.47**
302	8	0.71	44	1.11	0.64
315	13	0.46	75	0.30	1.53
326	19	1.38	99	1.10	1.25
334	17	0.43	161	0.45	0.95
250, 14 days	21	0.41	109	0.44	0.93
250, equivalent angles	21	143.95	109	95.29	1.51
261, 14 days	23	0.58	95	0.12	4.83**

These results show that in general under these circumstances no significant differences in stand can be demonstrated and thus there is little evidence that seed treatments differ in their ability to control post-emergence damping off expressed on the absolute basis of number of seeds planted. However, it should be pointed out that percentage post-emergence damping off is frequently calculated on the basis of the number of plants emerging rather than on the number of seeds planted. This, despite the fact that the percentage damping off or conversely percentage survival so calculated, may give no indication of the actual number of plants surviving. Since the various phases of seed decay and damping off are probably merely different symptoms of the same disease it appears illogical to express the results on any basis other than number of seeds planted. Furthermore from a practical standpoint it is the final stand of seedlings that is of importance. The results above showing no significant difference in stand are also evidence of a constant amount of post-emergence damping off in any given test for all treatments. Thus while 20 per cent of the seed planted might damp off in the control, about 20 per cent would also be expected even in the best treatments.

It is thus concluded that since seedling height is a function of stand, and stand is a function of emergence, it is only necessary to take records on emergence. Emergence being practically concluded by the 14th day, final records may be taken then with considerable saving in time, space, and labor.

DEPTH OF PLANTING

The possible effect of depth of planting in infested soil on emergence of seedlings was studied. In one test untreated seeds and seeds given a low dosage of Spergon were planted at depths of 1/4, 1/2, 3/4 and 1 inch. Observations were taken at intervals and are summarized in Table III A. While emergence was increasingly delayed with greater depths of planting, by the 14th day no difference could be shown in percentage emergence.

TABLE III

EFFECT OF DEPTH OF PLANTING ON PER CENT EMERGENCE OF SEEDLINGS IN INFESTED SOIL

Depth in inches	Days after planting			
	4	7	14	21
	A			
1/4	30	49	53	53
1/2	6	48	52	53
3/4	1	49	53	54
1	0	35	53	53
	B			
1/4	69	77	80	80
1/2	76	84	84	84
1	62	84	86	86
1-1/2	2	74	77	77
2	0	75	76	76

The effect of greater depths up to two inches was then studied. Here seed treated lightly with Arasan and Semesan was employed. The average results are given in Table III B where the same general effect is noted, a retarding in emergence, but no significantly demonstrable difference by the 14th day after planting.

Unless care is taken, however, pea seed may become uncovered at shallow depths, while covering to extreme depths is unnecessarily laborious.

Accordingly a depth of 1 inch, i.e. covering with four 4-inch pots of soil per small flat, was employed thereafter.

INOCULUM POTENTIAL OF INFESTED SOIL

It is desirable to be able to dilute the infested soil with fresh soil both to increase the amount of available infested soil and also to avoid any appreciable accumulation of toxic chemicals in the soil. Insofar as the dilution is just prior to planting seed it is necessary to know the effect of dilution on inoculum potential and hence on emergence. Horsfall (6) has defined the inoculum potential essentially as the disease producing power of the soil. In practice it may be measured by the percentage failure of viable seeds to emerge.

Infested soil was diluted by thorough mixing with various quantities of sterile soil so that preparations were available representing entirely infested soil, 1/2, 1/4, 1/8, etc. infested soil and entire sterile soil. The different lots were placed in separate replicated flats and sown with treated and untreated seed. The experiment was run three times at widely separated intervals so that the stock infested soil was not identical, at least it differed to the extent that repeated exploratory testing, cropping and composting might induce changes. The average results with Arasan at a low dosage of 0.06 per cent and with a check of untreated seeds are shown in Figure 6. It will be seen that on an arithmetic basis (Fig. 6 A) there is

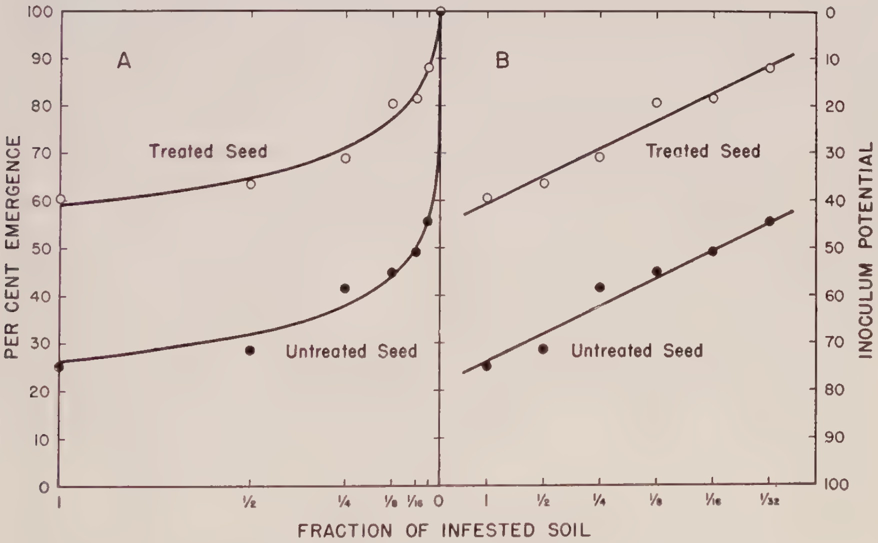

Figure 6. Effect of soil dilution on inoculum potential as measured by emergence of treated and untreated seed; 250 seeds per observation. A. Arithmetic graph showing J curves. B. Soil dilution plotted on semi-logarithmic graph paper showing linear relation.

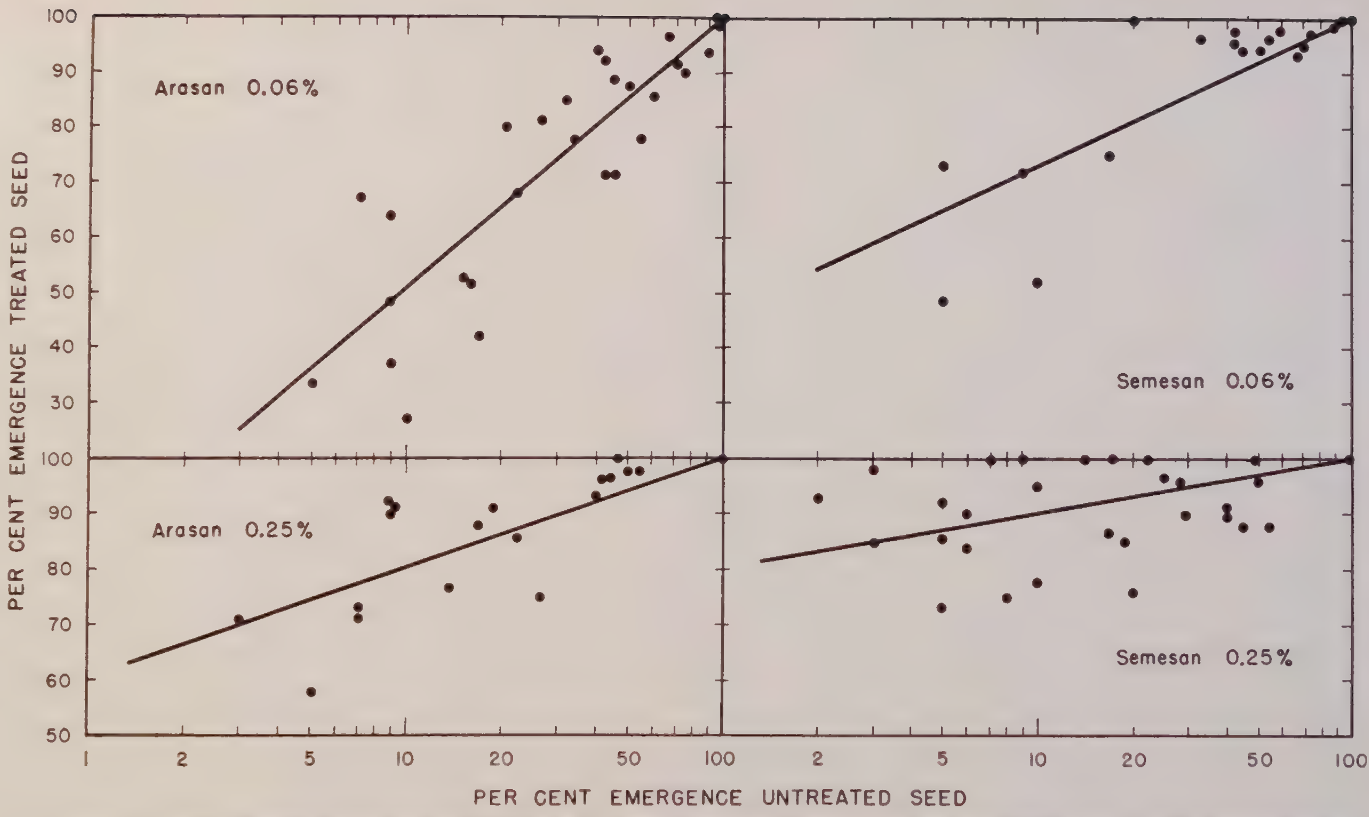

FIGURE 7. Linear regression of per cent emergence of treated seed on logarithm of per cent emergence of untreated control seed. Note slope is a function of chemical and decreases with increasing dosages. Regressions calculated to pass through 100 per cent point. Each observation on 30 to 100 seeds.

little change in inoculum potential up to a dilution of 1/4th. Actually the relation is logarithmic with the emergence increasing and the inoculum decreasing with the logarithm of the soil dilution (Fig. 6 B). In practice the infested soil was usually employed at a dilution of from 1/2 to 1/4 and a good grade of greenhouse soil was used as the diluent rather than sterile soil. Greenhouse soil is to be preferred to sterile soil for this purpose since it has a low inoculum potential of its own, and does not have a slight delaying effect on emergence associated with sterile soil.

It has been shown by Horsfall (7)[7] that the relation of protective value, i.e. emergence to inoculum potential may be expressed by a J curve concave downwards. From an accumulation of data this relation has been confirmed. However, a further study shows that there is a linear relation between the per cent emergence of treated seed and the logarithm of the per cent emergence of the check seed as illustrated in Figure 7 for two dosages of Arasan and Semesan. That is, the emergence of treated seed increases as the logarithm of the check seed emergence. Further, the slope of these curves is a property of the particular treatment and decreases with increasing dosage. Higher and more effective dosages of the same chemical have flatter curves expressing this relationship. Actually, for medium to high dosages of effective protectants it is difficult to observe any change in emergence of treated seed with changes in the inoculum potential.

DOSAGE-RESPONSE CURVES

The dosage-response curves were determined for the four standard materials used in this study. These may be considered typical for efficient seed protectants. The dosage ranged from 2 per cent of seed weight to 0.0156 per cent, with a dosage ratio of 2. The test was run twice, two years apart, with 60 seeds planted at each dosage and test. The relative results for the two tests agreed well and the mean values are plotted in Figure 8. It will be noted that, like foliage diseases in the greenhouse and field, the curves are very flat. In both tests Yellow Cuprocide showed a curve breaking at a dosage of 0.125 per cent to give a very flat response at the higher dosages. Due to the difference in slope of the four standard fungicides they vary in relative effectiveness at different levels of emergence. Some ED values[8] graphically calculated from these data are given in Table IV.

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[7] Dr. Horsfall states in correspondence that the designation, x and y axis should be reversed in his text (7, p. 22) and that in his Figure 8 the y axis should read "Emergence protected seed."

[8] The symbol ED for effective dose as defined by Finney (5, p. 18) is more general than the restricted LD or lethal dose. Hence, the former is more appropriate for use in most fungicide studies where the percentage response measured is non-germination of spores, disease control, emergence of seedlings, etc.

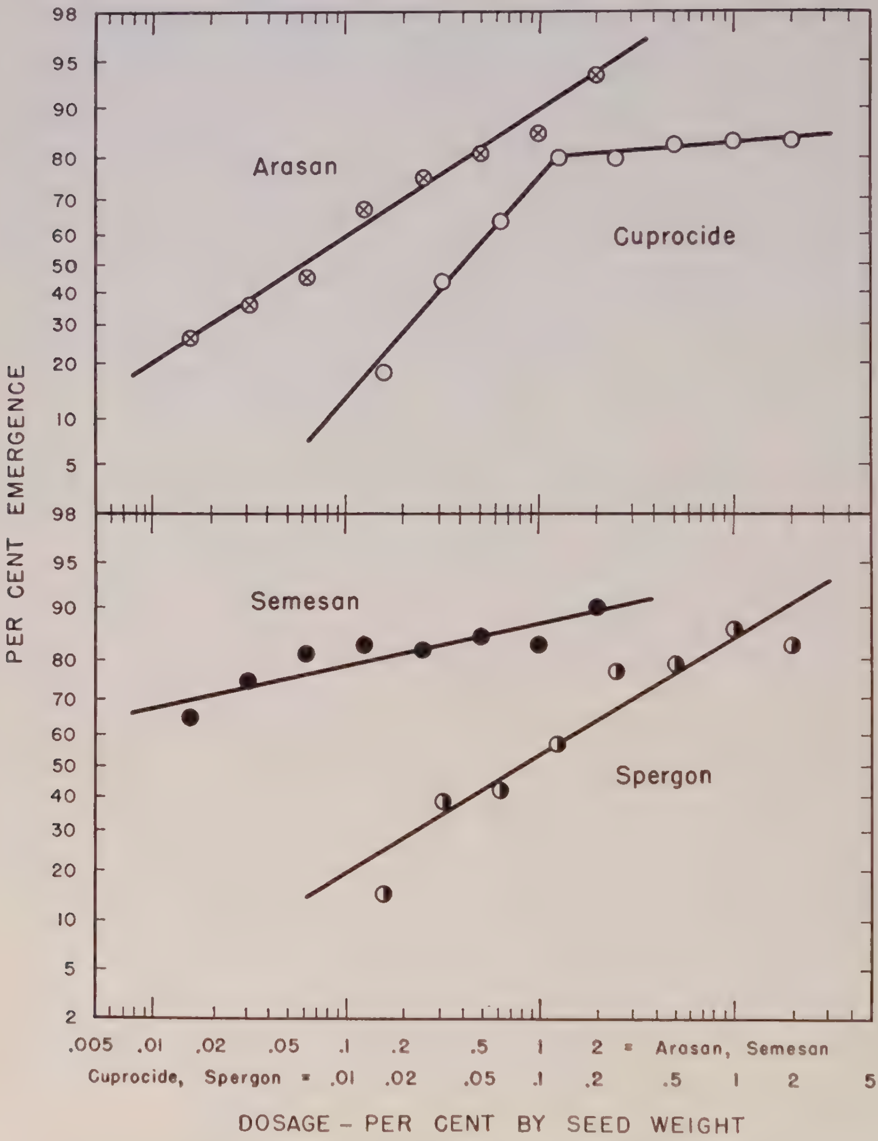

FIGURE 8. Dosage-response curves for pea seeds treated with four standard fungicides, 120 seeds per observation. Note overlapping scale, i.e. 1.0 per cent dosage for Arasan and Semesan coincides with 0.1 per cent for Cuprocide and Spergon.

TABLE IV

Difference in Relative Effectiveness of Standard Fungicides at Different ED Levels. Per Cent Dosage by Seed Weight

Fungicide	ED50	ED80	ED90
Arasan	0.06	0.50	1.1
Yellow Cuprocide	0.04	0.13	>4.0
Semesan	<0.01	0.15	2.3
Spergon	0.08	0.65	1.8

EFFECT OF PEA SEED COLOR

It was noted that the Perfection peas range in color from light green to cream,[9] and it was thought possible that the different colored seeds might differ in their susceptibility to seed decay and pre-emergence damping off. A sample of seeds was divided into two lots, the one containing green and mostly green colored seeds and the other cream and mostly cream colored seeds. Ten flats were planted with alternating rows of the two color types of seeds which were not treated. Results taken at 7 and 14 days on per cent emergence were as follows:

	7 days	14 days
Cream peas	5.7	10.7
Green peas	13.0	27.7

These differences are highly significant.

Since about a quarter of the seeds could be classified as all green, a quarter all cream and a half intermediate, another sample was made up of seeds in equal number from the three categories. These were planted untreated in flats of infested soil of different inoculum potentials. There were five flats of each soil, planted to two rows of each seed color or a total of 100 seeds of each color for each soil. The results at 14 days (continued observations to 30 days showed no appreciable change) are given in Table V. An analysis of variance showed high significance for pea color and for soils.

TABLE V

Per Cent Emergence of Untreated Pea Seeds of Different Color in Soils of Different Inoculum Potential

Pea seed color	Soil 1	Soil 2	Soil 3	Average soil
Green	48.0	52.0	60.0	53.3
Intermediate	18.0	33.0	34.0	28.3
Cream	6.0	15.0	19.0	13.3

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[9] Dr. Floyd L. Winter states in correspondence that this variety is normally a green cotyledon type and that the cream coloring is probably due to overmaturity at harvesting, or possibly strong sunlight or some other environmental factor.

However, the interaction of color and soil was not significant. There is a fairly constant ratio in susceptibility of the pea seed of different colors to attacks of damping off fungi as measured by per cent emergence. Thus the green peas give about twice the emergence of the intermediate peas, which in turn give about twice the emergence of the cream peas. This ratio can be seen also in the first experiment when corrected for the intermediate group. This difference cannot be ascribed simply to differences in viability, for the composite sample approaches 100 per cent germination in sterile soil.

Since pea seed color is of such importance in emergence, more drastic conditions can be obtained in greenhouse tests of protectants by employing only the cream colored seed. Also under some circumstances the practical control of seed decay and pre-emergence damping off may be aided materially by planting only the green seed.

EXPERIMENTAL DESIGNS

Since it has been shown by Youden (15) that appropriate experimental designs may be used to increase the accuracy of greenhouse tests and further, since preliminary work with artificially infested soil has shown much heterogeneity, considerable attention was devoted to a study of experimental designs.

Over a period of time numerous different seed treatments were tested in small flats, large flats, and in narrow side benches. The basic unit as usual was 10 seeds per dosage and this was replicated from three to ten times. The experimental designs included especially, balanced incomplete blocks, and also randomized complete blocks, balance lattices and replicated triple lattices. These designs were obtained for the most part from Cochran and Cox (3). Usually the block consisted of an entire flat, though in a few cases it was half of a large flat or small area of greenhouse bench. Results were taken on both emergence and stand in many cases. It was thought that block difference might be expected to be greater on stand measurements due to the added factor of damping off.

A summary of results is given in Table VI A for 10 different tests. It will be noted that, as expected, results taken on stand tend to show greater heterogeneity than those on emergence. However, in only two cases was there a significant block or flat variation for stand data and only one for emergence. Thus it is concluded that in general differences between flats are insignificant. Hence there is no need to use complex experimental designs such as the balanced incomplete block in order to have small homogeneous blocks consisting of a single flat.

Hereafter most tests were run as randomized complete blocks, with the block consisting of a number of flats. That is, the various treatments were randomized in a block consisting of the required number of flats.

Some results with this type of design are summarized in Table VI B. It will be seen that in all cases but one the block effect was insignificant.

The marked exception of Test 360 was due to the higher emergence in the third block. The blocks here were unusually large with 33 flats and the test required two days to plant with the soil for the three blocks being mixed at different times. Presumably, this resulted in a lowered inoculum potential for the last block.

TABLE VI

Non-Significance of Block or Flat Variation on Emergence or Stand

A. Blocks consisting of single flat or other small area

Test No.	Emergence or Stand	Blocks D.F.	Blocks M.S.	Error D.F.	Error M.S.	F Value & significance
250	E	30	2.55	125	1.93	1.32
250	S	30	3.25	125	2.97	1.09
253	S	20	1.61	64	1.33	1.21
256	E	24	3.02	96	3.36	0.90
256	S	24	3.73	96	3.88	0.96
269	S	30	1.70	125	1.63	1.04
285	S	15	2.07	65	1.45	1.43
289	S	20	1.73	65	1.84	0.94
302	E	30	1.55	125	1.41	1.10
302	S	30	1.76	125	1.50	1.17
315	E	15	2.88	65	1.90	1.52
315	S	15	3.76	65	1.54	2.44**
326	S	30	1.92	145	1.28	1.50
334	E	18	3.02	153	1.74	1.74*
334	S	18	4.81	153	2.09	2.35**

B. Block consisting of multiple flats. Emergence data only

Test No.	Number flats in block	Blocks D.F.	Blocks M.S.	Error D.F.	Error M.S.	F Value & significance
360	33	2	49.63	198	3.51	14.14**
364	10	9	4.87	531	2.59	1.88
382	18	4	1.44	84	2.23	0.65
386	6	4	1.37	24	3.86	0.35
390	2	12	3.15	100	2.60	1.21
409	14	2	1.16	148	3.51	0.33

In general, randomized complete block arrangements using multiple flats should prove satisfactory and any block effect if present may be removed if desired.

SIGNIFICANT DIFFERENCE BETWEEN TREATMENTS

Precision of Replicates

The basic group in all these tests is the replicated 10 seed row unit, and it was considered of interest to determine the precision of replicates within a given test. That is, do replicates vary more than is to be expected from errors of random sampling? The appropriate examination here is the χ^2 test; however, units of 10 seeds are too small for suitable application. Accordingly data were collected on tests where 10 replicates were used, i.e. 100 seeds. The 10 seeds in each replicate were selected at random, as usual, regardless of color. These 100 seeds were arbitrarily divided into two groups consisting of the first five replicates totaling 50 seeds and the second five totaling 50 seeds. Paired sets of the 50 seeds, comprising emerged and non-emerged seeds, for a given treatment and test were then analyzed by the χ^2 test (13). The summarized data are given in Table VII. It will be seen that the different tests are uniform and that a total n of 50 and χ^2 of 50.62 results in a probability or P value of 0.35. This very reasonable probability indicates that the replicates within a test do not vary more than is to be expected from the variation of random sampling and that the technique of a single test cannot be appreciably refined.

TABLE VII

χ^2 TEST ON PAIRED REPLICATES OF 50 SEEDS; EMERGED AND NON-EMERGED

Test	n	χ^2	Probability
204	14	16.01	.30–.20
267	6	4.56	.95–.50
347	8	8.71	.50–.30
364	22	24.18	.50–.30
Total	50	53.46	.35

Comparison of Treatments

It has been shown in previous studies on methods of evaluating fungicides (12) that greater dependability may be placed on comparisons of treatments run at different times or tests rather than at one time as is commonly done. It was also shown that advantage may be taken of previous experience by means of cumulative error terms (11) obtained from similar kinds of tests and that those error terms with their larger degrees of freedom may be used provided the homogeneity test is met.

Data accumulated over a period of time on emergence tests with standard and other fungicides repeated at the same dosage gave the following error terms:

	Degrees freedom	Mean square
Replicate 10 seed rows	857	2.13
Treatments × tests	82	4.01

As is to be expected, the difference between these is highly significant although earlier limited comparisons mostly with high emergence standard fungicides indicated no difference (10).

From these representative data it is possible to construct a table showing the significant difference required, at various numbers of replicate rows and tests, for comparing treatments. In preparing this table the variance components were estimated by the method of Crump (4). The computed required differences are given in Table VIII where it will be observed that, for example, a 27 per cent difference would be significant for three replicate rows, i.e. 30 seeds tested once. However, this required difference may be reduced to 11 per cent by testing 10 replicate rows at three different times. It should be appreciated that these are very small samples; in a large scale test relatively small differences would be significant.

TABLE VIII

PER CENT LEAST SIGNIFICANT DIFFERENCE BETWEEN PEA SEEDS TREATMENTS. ODDS 19:1

Number of tests	Number of replicated 10 seed rows							
	1	2	3	5	10	20	50	100
1	42.7	31.9	27.3	23.0	19.1	16.8	15.3	14.7
2	30.2	22.6	19.3	16.2	13.5	11.9	10.8	10.4
3	24.7	18.4	15.8	13.3	11.0	9.7	8.8	8.5
5	19.1	14.3	12.2	10.3	8.5	7.5	6.8	6.6
10	13.5	10.1	8.6	7.3	6.0	5.3	4.8	4.7

EXPERIMENTS WITH OTHER SEEDS

Some studies were made with treatments on various other seeds to determine how they would compare with the pea as a possible standard seed for preliminary evaluation tests. The seeds were beet (*Beta vulgaris* L.) vars. Extra Early Egyptian and Detroit Dark Red; bush bean (*Phaseolus vulgaris* L.) var. Tendergreen; cucumber (*Cucumis sativus* L.) vars. Black Diamond and Davis Perfect; lima bean (*Phaseolus limensis* Macf.) var. Fordhook Bush Lima; peanut (*Arachis hypogaea* L.) var. Spanish; radish (*Raphanus sativus* L.) var. Scarlet Globe; spinach (*Spinacia oleracea* L.) var. Bloomsdale Long Standing; and tomato (*Lycopersicon esculentum* Mill.) var. Bonny Best.

The seeds were handled in the same manner as the peas, being treated the same way with a similar dosage series except that the beet seed dosage was one step higher. The seeds were planted, 10 seed row units, in the infested soil, with the larger seeds being sown deeper than the peas and the smaller ones shallower. Large flats were necessary for the lima beans, but small flats were used for all others. Observations were taken at intervals on emergence and stand.

Emergence and stand. Covariance analyses were run on beet, cucumber, lima bean, peanut, and spinach data and in all cases but lima bean no significant difference could be shown between the stands of different treatments when adjusted to a common emergence level. In the case of the lima bean data an examination showed an unusually large amount of damping off in mercury-containing treatments which was presumably associated with mercury injury to the seeds and seedlings. When these treatments were removed from the analyses no significant difference could be shown. Thus, as with peas, there is no evidence that treatments differ in their ability to control post-emergence damping off.

Flat variation. Experiments with beets, cucumber, peanut, and spinach were designed to show possible flat variation. In the case of the first three kinds of seed no appreciable variation could be shown, while with spinach (one test) a significant difference was found. It is probable that in general, as also with peas, flat variation is relatively insignificant.

Replicate row error. In a number of tests with all the different seeds the replicate row error was found to approximate that of peas, with the exception of beets. Since beet is a multiple seed it is to be expected that the variation would be greater. With this exception, the precision with these seeds can be expected to be similar to that of the peas, and the necessary differences for significance given in Table VIII are indicated.

Specificity. The only specific responses noted for the different seeds toward different chemicals was the well known injuriousness of copper to cruciferous seed, e.g. radish, and the marked sensitiveness of lima beans to mercury as mentioned above.

General considerations. It is to be emphasized again that these comparisons are with the view to selecting seeds most suited to initial and general screening tests. However, if there is special interest in selecting a seed protectant for some particular crop, that seed naturally will be used regardless. One criterion for choosing a seed for general purposes is ease of handling. Some seeds are too small and hence difficult to handle when planting; also large numbers are needed to provide enough for a weighable amount of chemical. Tomato seed falls in this class. Again some seeds are too large and require undue amounts of chemical and planting space; such a seed is lima bean. The control seeds must give a low percentage emergence; that is, the inoculum potential of the soil for the specific seed must be high. In these tests bush bean, peanut, radish, spinach, and tomato tended to give too high an emergence for best comparisons. Multiple seeds such as beet are unsatisfactory because of variation in the individual number of viable seeds per row. Seeds highly susceptible to specific injury such as crucifers and lima beans respectively to copper and mercury are unsuitable for general purposes. Cucumber seed was generally satisfactory, though high emergence generally resulted from relatively low dosages. It may be considered a second choice to peas.

TESTS WITH A GROUP OF CHROMATE COMPLEXES

In order to demonstrate how this method works in practice a description is given of some typical tests with a number of related chemicals. A group of 157 inorganic and metallo-organic chromate complexes and related materials kindly furnished by the Carbide and Carbon Chemicals Corporation on the basis of slide-germination tests (1) and other information were selected for investigation as possible seed protectants. The procedures developed above were followed throughout.

First Test

All the chemicals were tested against peas in three replicated rows in complete randomized blocks at a dosage of 0.0625 per cent. A very few compounds were also run at 0.0156 per cent. The standard fungicides were Arasan, Semesan, Spergon, and Yellow Cuprocide at dosages of 0.25, 0.0625 and 0.0156 per cent. This gave a series of 198 different treatments which required 99 flats plus 1 flat of sterile soil. A view of the test (No. 360) at 14 days is shown in Figure 9. The control gave 7 per cent emergence so the test would be considered a severe one. The results showed varying degrees of emergence and the 18 most promising compounds were selected for further study.

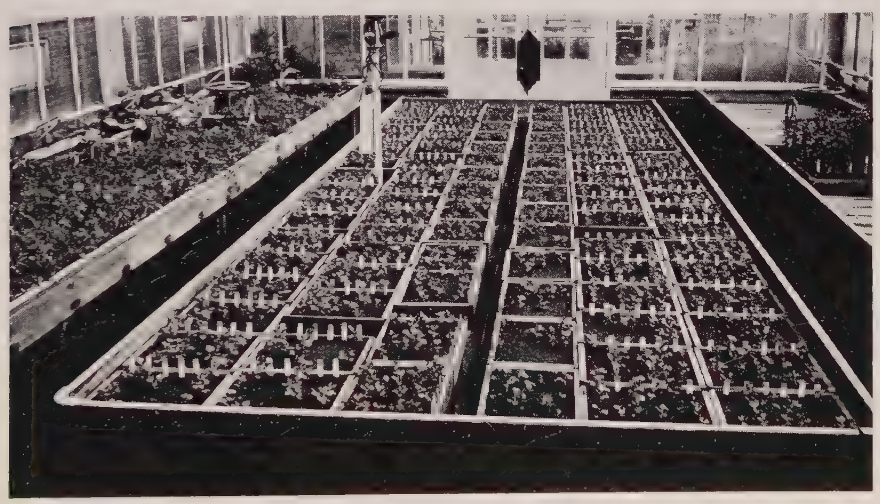

FIGURE 9. Initial test on a series of 198 different treatments (dosage 0.0625 per cent) replicated three times in 10 seed row units; 14 days after planting.

Second Test

These 18 complexes together with the mercury and copper standard fungicides, namely, Semesan and Yellow Cuprocide, were tested against peas in more detail. Three dosages, 0.25, 0.0625 and 0.0156 per cent were used with 10 replications for each dosage, i.e. 100 seeds per dosage and 300 seeds per chemical. Each set of replicates constituted a randomized block. However, for a possible more precise comparison of dosages, all three for a given chemical were placed at random side by side in one-half of a flat; the other half contained another treatment. This test also consisted of 100 flats.

The results of this test are given in Table IX. The percentage dosage estimated for 80 per cent emergence is also recorded. This value was obtained graphically by plotting on logarithmic-probability paper as shown for the better preparations in Figure 10. Since this was an unusually severe test with control showing only 5 per cent emergence and since only medium and low dosages were employed, the data were more suitable for estimating the ED80 rather than a higher ED level. Incidentally, the ED80 was later calculated by the method of Bliss (2) and the estimates were found to agree within an average of 3 per cent, the greatest departure being 10 per cent. This substantiates the evidence advanced in an earlier paper (12) in favor of using the simple graphic estimate. The least significant difference as

TABLE IX

PER CENT EMERGENCE OF CHROMATE COMPLEXES

Code No.	Chromate type	Per cent dosage			Per cent dosage for 80% emergence (ED80)
		0.25	0.0625	0.0156	
202	Hg	56	83	52	Injured
222	Hg Cu	81	71	43	0.20
224	Hg Zn	81	57	37	0.26
9	Cu	75	36	34	0.78
53A	"	57	46	44	>2
298	"	57	38	16	1.2
323	"	73	47	31	0.51
440	"	45	37	33	>2
519	"	69	42	28	0.75
169E	Cu Zn	41	34	24	>2
245	"	56	40	32	>2
257	"	64	36	21	0.90
640	"	74	44	25	0.43
518D	Cu Cd	51	37	31	>2
170	Cd	50	32	37	>2
153	Misc.	39	33	18	>2
179	"	43	29	26	>2
664	"	42	23	6	2.0
Semesan		84	48	39	0.28
Yellow Cuprocide		79	46	27	0.31
Control			5		
Sterile Soil			98		

Least significant difference: 1 dosage 14%, mean of 3 dosages 8%.

calculated for this single test (Table IX) is comparable with that for the method in general (Table VIII) especially when it is realized that the latter contains the greater variation due to the component treatment × test interaction. It will be observed that two complexes, Nos. 222 and 224, compare favorably with Semesan and Yellow Cuprocide while a third, No. 640, is only slightly less effective in this test. Compound No. 202, however, shows marked injury. There was indication that No. 222 gave some injury as shown by the amount of damping off at the highest dosage. Results with No. 224 in comparison with Semesan and controls for typical individual flats are shown in Figure 2. In the third flat from left to right there is: Semesan, at 0.25, 0.0625 and 0.0156 per cent dosages, and No. 224, with the same order of dosages.

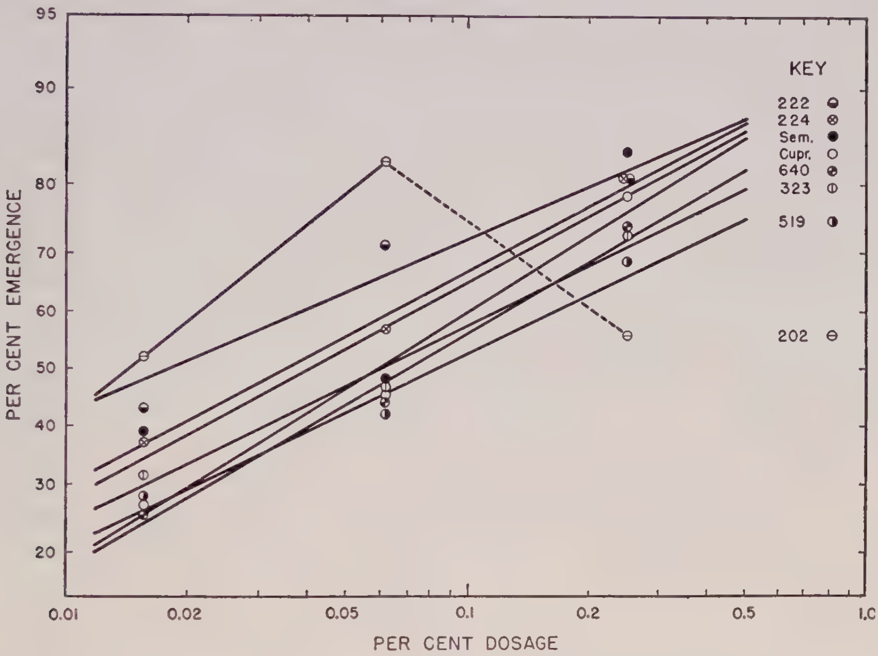

Figure 10. Dosage-response curves plotted on logarithmic-probability scale for experimental chromate fungicides in comparison with two standards. Note injury expressed as reduced emergence for No. 202 at the 0.25 per cent dosage.

Tests with Other Seeds

As the final step in the preliminary greenhouse evaluation of these new chemicals as seed protectants the six most interesting materials were tried on seeds of beet, bush bean, cucumber, lima bean, radish, and spinach, in comparison with Semesan and Yellow Cuprocide. Dosages of 1.0, 0.25, and

0.0625 per cent were applied in all cases except beet where 4.0, 1.0, and 0.25 per cent were used. The middle dosage in all cases is approximately equivalent to that recommended for standard commercial protectants. The dosages were replicated five times, i.e. 50 seeds per dosage and 150 seeds per chemical for each different kind of seed.

TABLE X

PERCENTAGE EMERGENCE AT 14 DAYS OF VARIOUS SEEDS TREATED WITH CHROMATE PREPARATIONS IN COMPARISON WITH SEMESAN AND YELLOW CUPROCIDE AT 3 DOSAGES. 50 SEEDS PER DOSAGE

Beet

Preparation	4.0%	1.0%	0.25%	Control	Sterile soil
Chromate 224	125	130	108	78	107
Chromate 640	115	142	123		
Semesan	140	118	127		
Yel. Cuprocide	137	132	92		

Bush bean

Preparation	1.0%	0.25%	0.0625%	Control	Sterile soil
Chromate 224	74	88	86	72	86
Chromate 640	74	84	78		
Semesan	80	88	74		
Yel. Cuprocide	90	80	80		
Cucumber					
Chromate 224	98	100	96	22	98
Chromate 640	98	100	98		
Semesan	100	98	90		
Yel. Cuprocide	96	100	98		
Lima bean					
Chromate 224	0	0	12	5	78
Chromate 640	54	58	48		
Semesan	0	0	46		
Yel. Cuprocide	46	38	32		
Radish					
Chromate 224	92	84	84	83	88
Chromate 640	60	72	80		
Semesan	80	88	80		
Yel. Cuprocide	62	62	78		
Spinach					
Chromate 224	74	68	52	55	66
Chromate 640	74	60	58		
Semesan	62	58	56		
Yel. Cuprocide	74	62	50		

Against beet, bush bean, and spinach medium control emergence resulted and all materials gave good protection to beet and bush bean and fair protection to spinach, so that no significant differences could be shown between any of the experimental materials or standards. Likewise with cucumbers all materials gave excellent emergence even though the controls were low, and no differences could be shown.

Radish seed as expected showed injury from all the copper-containing chromates as well as Yellow Cuprocide. However, complex No. 224 gave control of seed decay equal or superior to Semesan.

Lima beans being susceptible to mercury showed pronounced injury to the mercury-containing complexes, Nos. 222, 224, and Semesan. Chromate complex No. 640 was without injury and more effective than Yellow Cuprocide.

The results with chromates Nos. 224 and 640 in comparison with Semesan and Yellow Cuprocide are recorded for all seeds in Table X.

Conclusions

By a series of eliminating steps two chromate complexes, out of 157 initially tested, have shown promise as seed protectants in comparison with standard fungicides. These chromates are: No. 224, $7ZnO \cdot 2HgO \cdot 2CrO_3 \cdot 7H_2O$ and No. 640, $ZnO \cdot 4CuO \cdot CrO_3 \cdot XH_2O$. With this background these two materials are now considered suitable for testing under practical field conditions as seed protectants against a variety of crops and locations in comparison with the best standard fungicides.

SUMMARY

1. Quantitative studies were made of various factors influencing the preliminary evaluation of chemicals as possible seed protectants by greenhouse tests on peas. The variety Perfection, highly susceptible to seed decay and damping off, was used. Seeds were treated with various standard and experimental fungicides as dusts with one or more dosages from the series 4.0, 1.0, 0.25, 0.0625, and 0.0156 per cent of seed weight, rolled in specimen bottles and planted in flats of naturally infested soil. The basic unit was a row of 10 seeds for each dosage. The row was replicated.

2. Observations were taken at varying times on emergence, stand (difference between emergence and stand is post-emergence damping off), and height of seedlings. Emergence is practically completed by the 14th day, though stand continues to diminish with time. There is a highly significant regression of height on stand, hence little is to be gained by recording height.

3. By covariance analyses it was established that when stand is adjusted to a common emergence level there was in general no significant difference between stands, i.e. treatments did not differ in their effectiveness in

controlling post-emergence damping off, expressed on basis of number of seeds planted. Hence damping off is probably independent of treatment. There is no need to record stand, and results may be taken on percentage emergence at 14 days. Increasing the depth of planting delayed but did not change final emergence.

4. After use, infested soil is composted and eventually diluted with fresh greenhouse soil for use again. There is little change in inoculum potential up to a dilution of 1/4. Emergence increases and inoculum potential decreases with the logarithm of the soil dilution. The emergence of treated seed increases as the logarithm of the control seed emergence. The slope of these curves is a property of the particular treatment and decreases with increasing dosage.

5. Most chemicals appear to give a straight line response when plotted on a logarithmic-probability scale. The slopes are flat but vary for different compounds.

6. Perfection peas range in color from light green to cream. Cream peas are twice as susceptible to decay as are intermediate colored ones, and four times as susceptible as green peas.

7. Numerous tests with various types of experimental designs, in general, failed to show a significant individual flat variation. Complete randomized block arrangements of multiple flats are suggested. The precision of replicated samples of 50 treated seeds within a given test was found by the χ^2 measure not to vary any more than that reasonably to be expected of random sampling. Hence the technique of a single test cannot be appreciably refined.

8. A table of significant differences is presented, compiled from cumulative errors of the two components treatment × test interaction and replicate 10 seed row. A difference of 27 per cent is necessary for two treatments tested once with three replicated rows, i.e. 30 seeds each. For three tests and 10 replicates, an 11 per cent difference is required.

9. Limited experiments performed with seeds of beet, bush bean, cucumber, lima bean, peanut, radish, spinach, and tomato, showed, in general, no significant difference between stands by covariance, nor between flats. Replicate row error approximated that of peas. Radishes were sensitive to copper injury and lima beans especially so to mercury. For an ideal method some seeds are considered too small (tomato) and some too large (lima bean) while multiple seeds (beet) are also unsuitable.

10. Exemplifying this procedure is a series of tests with 157 chromate complexes. Following a first test at a dosage of 0.0625 per cent on 30 seeds, 18 materials were run in a second test at three dosages (100 seeds) in comparison with Semesan and Yellow Cuprocide. Six were then selected for trial on beet, bush bean, cucumber, lima bean, radish, and spinach. Two compared favorably with the standard fungicides and are considered

worthy of practical field tests. These are: No. 224, $7ZnO \cdot 2HgO \cdot 2CrO_3 \cdot 7H_2O$ and No. 640, $ZnO \cdot 4CuO \cdot CrO_3 \cdot XH_2O$.

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EFFECTIVENESS OF CERTAIN APPLE POLLEN DILUENTS IN HAND POLLINATION TESTS

Norma E. Pfeiffer

HISTORY

There has been a marked development of hand pollination in apple (*Pyrus malus* L.) varieties in the years since MacDaniels and Heinicke (6) indicated in 1929 that such pollination might increase the set in blocks of self-sterile varieties, although there were as yet no practicable methods for work on any considerable scale. Nevertheless methods were briefly described which covered pollen collection and hand pollination with favorable conditions for each.

The following year MacDaniels (4) published the results of investigation into effective methods of applying pollen on a large scale and the use of diluents to make more economical use of the collected pollen. Such substances as wheat flour, cornstarch, powdered charcoal, Fuller's earth, Lycopodium, and dusting sulfur were mixed with apple pollen in proportions of two-thirds diluent to one-third pollen. When sowed on nutrient agar, all gave good germination of pollen except the mixture with sulfur, which had previously been shown (5) to decrease the percentage of germination of pollen. Further tests after 20 days showed the pollen to be still viable, although fewer tubes were produced. However, when diluted pollens were applied to stigmas, only the mixture with Lycopodium gave a satisfactory set. These experiments were considered preliminary and hand pollination was not recommended as "either practical or desirable under most conditions."

However, large scale trials were made in Delicious and Winesap orchards of the Northwest soon after this (3) which were followed by the report of Overley and Overholser (8) on hand pollinating Delicious and Winesap blossoms with Jonathan pollen. Comparison of set was made between open-pollinated branches and those with hand pollination in addition to pollination due to insects, with no blossoms covered. Although results were variable, the evidence of increased set even when only one flower to every tenth cluster was hand pollinated showed the value of the method. Increase of acreage in which hand pollination was practiced from 500 acres in 1935 to 1,000 to 1,500 acres in 1937 in two Washington districts reflected the interest of the orchardist.

By 1942, Antles (1) in a brief article reported that at least 4,000 acres

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of apples were hand pollinated the previous year. Snyder in 1946 (9) said that hand pollination as a supplementary device was past the experimental stage. In 1947, Overley and Bullock (7) reviewed the whole situation and brought it down to date with consideration of methods of application by hand, hand dusters, water spray, airplane, and bomb[1], with the first one proving most dependable. Experimenting with growth chemicals which have been found to decrease fruit set and with thirteen inert substances, they found Lycopodium the most satisfactory diluent and talc least, with no set. Lately Antles (2) has received a patent on "method of treating and applying live pollen" with the use of *Lycopodium clavatum* spores as a carrier.

METHODS AND RESULTS

At the time of undertaking the present work, the author was unaware of some of the previous work on this phase. Therefore some of the tests represent a repetition, since essentially the same methods were followed, first of germination of pollen with and without diluents on hanging drops of nutrient agar, and second of application of similar samples on stigmas of apple flowers. Comparison of the fruit set resulting from the use of pollen with different diluents indicates their relative effect on germination of the pollen on the stigma.

Flowers of Stayman Winesap trees were used for pollination. Branches which could be reached from the ground were bagged with cheesecloth bags the week before blooming. Because of limited plant material, all flowers on each spur within each bag were hand pollinated. There was no emasculation in the blossoms to be pollinated because it was anticipated that Stayman Winesap would be self-sterile. However, for certainty, flowers on four bagged branches on three trees were selfed in the following numbers: 17, 14, 11, 13. In no case in the 55 pollinations was any fruit set.

A supply of Jonathan pollen (labeled A here) was received April 12, 1946, from the Division of Fruit and Vegetable Crops and Diseases at Beltsville, Md. This had been collected at "balloon" stage of the flower, separated by rubbing the anthers on a brass screen, and cured for 72 hours at 70°F. before shipping. A second lot of Jonathan pollen (B) was collected locally on April 25.

The following diluents[2] were used in mixtures of one-half diluent and one-half pollen (anther walls not removed): 1. almond shell flour; 2.

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[1] The bomb, of which several thousand are reported to have been sold and used in 1946, is constructed of "a heavy cardboard cylinder with a fused impelling charge of powder in the base. The cylinder was filled with talc as a carrier for the pollen. The pollen was placed on top of the talc, just prior to placing the bomb in the tree or on the ground to explode" (7, p. 166).

[2] Supplied by Mr. Kenneth S. Karsten (then of Niagara Sprayer and Chemical Co.) who made the pH determinations of suspensions of the first six.

kaolin; 3. carbola talc; 4. Eastern Magnesia 23 talc (EM 23 talc); 5. Pyrax ABB; 6. wheat flour; and finally, 8. Lycopodium powder was added to the list. Undiluted pollen is in the seventh place in the sequence. Each pollen collection was used with the seven carriers.

Pollinations were made using the mixtures with pollen A on April 22, 23, 25 and 30, with pollen B on April 30, May 1 and 2. Both lots were used on different flowers of the same tree, with similar distribution on center and lateral flowers for the mixtures. An endeavor was made to pollinate similar numbers of flowers except insofar as preliminary results on germination of Jonathan pollen stored the previous year with diluents influenced the number tested with almond shell flour. Table I gives the data concerning pollen stored April 20, 1945; a week later the various diluents were added, and the whole returned to storage over a saturated solution of calcium chloride in a desiccator held in an automatic refrigerator at about 40°F. In subsequent tests for viability, the pollen with diluent was sowed in duplicate on a medium containing 10 per cent cane sugar and 1 per cent agar. The germination percentages showed the mixtures with four carriers to give approximately as good results as undiluted pollen, namely kaolin, EM 23 talc, Pyrax ABB and wheat. The other two were poor, carbola talc with low germination and almond shell flour with none. This 1945 pollen was not used in the orchard.

TABLE I

EFFECT OF DILUENTS IN MAINTAINING VIABILITY OF APPLE POLLEN STORED OVER A SATURATED SOLUTION OF $CaCl_2$ AT 5° C., STARTING APR. 20, 1945

Diluent	Per cent germination on		
	May 18 1945	Jan. 23 1946	Feb. 8 1946
Almond shell flour	30 40	0 0	0 0
Kaolin	64 73	33 43	34 44
Carbola talc	37 46	17 20	13 18
Eastern Magnesia 23 talc	62 65	35 47	44 51
Pyrax ABB	58 61	44 49	45 48
Wheat flour	67 76	36 45	41 46
Undiluted pollen	82 84	43 51	44 48

Fruit set. Data are presented in Table II for the fruit set resulting from A and B collections of pollen with diluents, as determined on June 21 and at the end of the season on October 8. There were heavy losses along with heavy rains in early June, so that the June drop had occurred by the date selected. In October, the remaining fleshy fruits were brought into the laboratory, measured as to diameter, and examined for number of seed and cavities with seed. Some fruits which dried but remained attached were not included in the October data in Table II. Information is here given on the basis of size of fruit as well as number, partly because it is assumed that there was great competition where every flower was pollinated. With some exceptions, there is fairly good agreement between the results from pollen collections A and B with the different carriers. A comparison of the total

TABLE II

EFFECTIVENESS OF APPLE POLLEN DILUENTS IN HAND POLLINATION EXPERIMENTS

Diluent*	Pollen coll.	No. poll.	No. fruit set			Per cent fruit set		
			June 21	Oct. 8		June 21	Oct. 8	
				>3 Cm.	>5 Cm.		>3 Cm.	>5 Cm.
Almond shell flour	A	26	5	2	2	19	8	8
	B	29	3	0	0	10	0	0
	Sum	55	8	2	2	15	4	4
Kaolin	A	43	1	0	0	2	0	0
	B	29	7	1	1	24	3	3
	Sum	72	8	1	1	11	1	1
Carbola talc	A	40	2	1	1	5	3	3
	B	31	6	3	2	19	10	6
	Sum	71	8	4	3	11	6	4
Eastern Magnesia 23 talc	A	39	18	6	6	46	15	15
	B	31	16	14	10	51	45	32
	Sum	70	34	20	16	49	29	23
Pyrax ABB	A	40	3	2	1	8	5	3
	B	31	15	6	5	48	19	16
	Sum	71	18	8	6	25	11	8
Wheat flour	A	42	17	12	10	40	29	24
	B	30	12	5	5	40	17	17
	Sum	72	29	17	15	40	24	21
Undiluted pollen	A	35	22	8	7	63	23	20
	B	32	17	7	6	53	22	19
	Sum	67	39	15	13	58	22	20
Lycopodium powder	A	39	20	13	12	51	33	31
	B	29	16	9	5	55	31	17
	Sum	68	36	22	17	53	32	25

* Equal volumes of pollen (with anthers) and diluent.

TABLE III
COMPARISON OF THE TWO POLLEN COLLECTIONS USED

Observations	Total No.		Per cent	
	A	B	A	B
Pollinations	304	242	—	—
Fruit set June 21	88	92	29	38
Fruit >3 cm. Oct. 8	45	44	15	18
Fruit >5 cm. Oct. 8	38	34	13	14

set resulting from the two collections given in Table III indicates that A and B may be considered as similar. Therefore the combined data of the two collections are given in the line "Sum" for each diluent in Table II. In both June and October it is seen that these results with EM 23 talc, wheat flour and Lycopodium powder are in the same general range as with undiluted pollen. Results with the other four diluents are notably lower, indicating less favorable qualities as carriers.

Seed set. Counts of large seed, filled or not, and small seed were made for all fleshy fruits collected in October, whether of the minimum diameter indicated in Table II or not. The average number of seeds ranged from 8 to 10, except with kaolin and carbola talc, where it was 7; with only large seeds considered, these diluents also ran lower with 4 and 5 as an average against 6 or 7 with all other diluents. If one assumes that adequate and similar amounts of pollen were applied to the stigmas in the different pollinations with diluted pollen and calculates the average seed set per pollination with the different diluents in the order listed in Table II, the following results are obtained in the series: 0.6; 0.3; 0.5; 2.6; 1.0; 2.2; 2.4; 2.7. Here the diluents EM 23 talc, wheat flour and Lycopodium run in the same general range as the undiluted pollen, in seed production as in fruit production.

DISCUSSION

The number of pollinations here made is low as compared with the western orchard experiments. This may therefore be better considered as a preliminary for tests on a large scale, particularly with regard to the results with talc. MacDaniels (4) and Overley and Bullock (7) found that talc resulted in no fruit set. Our results indicate that there may be differences between talcs, since carbola talc gives poor results as a carrier compared with EM 23 talc. The pH value of suspensions in distilled water of carbola talc at 9.40 is nearer to that of EM 23 talc at 8.65 than it is to the other unfavorable carriers, almond shell flour, pH 4.70, kaolin 4.00, and Pyrax ABB 4.20. So pH value is not likely to be the important factor. Whether the different results with the talcs are based on different proportions of constituents (10), different particle size or some other factor is not known. In our stock, there were many coarser particles, more in the approximate size range of apple pollen, in EM 23 talc, while the carbola talc was much finer and more uniformly so. It might be worth while to experiment further with specific talcs, especially if bombs come to be important through improvement. Overley and Bullock (7) have indicated that talc because unsatisfactory as a carrier is not good in bombs. Lycopodium powder, recognized as an effective carrier by previous workers (1, 4, 7), is unsuitable for bombs because of its inflammable character which results in pollen injury.

The data in this experiment agree with these earlier results on Lycopodium spores. They apparently agree less well with regard to wheat flour reported to result in an unsatisfactory set (7) or none (4). In the former, the proportion of carrier was either three-fourths or one-half (not specifically given for wheat flour), in the latter two-thirds of the mixture. Whether our usually higher proportion of pollen or the size of particles in our particular sample played a role, we have no way of knowing. Possibly too, as less experienced operators, we applied pollen mixtures in greater amount. But as our results run, the wheat flour mixture was about as effective as EM 23 talc, Lycopodium and undiluted pollen as far as both fruit set and seed set were concerned.

SUMMARY

Of seven inert substances used to dilute Jonathan apple pollen before application to Stayman Winesap stigmas, the following gave fruit sets approximating that resulting from undiluted pollen: Eastern Magnesia 23 talc, wheat flour and Lycopodium powder. The last had previously been found excellent by other workers.

Four carriers including carbola talc, almond shell flour, kaolin and Pyrax ABB, gave unsatisfactory results.

It is suggested that inasmuch as talcs vary a good deal, larger scale experiments with Eastern Magnesia 23 talc and others not previously tested may bring out an inorganic carrier able to compete with Lycopodium spores. Bomb development calls for a diluent other than Lycopodium, which because inflammable injures the pollen in discharge of the bomb.

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A SIMPLE APPARATUS FOR ASSAY OF FUMIGANT INSECTICIDES

J. H. Standen[1]

Routine assay of a large number of chemicals requires rapid and dependable methods. The apparatus herein described has been found satisfactory over a three-year period for the testing of chemicals as fumigants.

The apparatus was designed primarily to test chemicals as fumigants for the control of such greenhouse pests as mealy bug and red spider. It consists of a series of heating coils by which weighed samples of materials may be vaporized in known volumes of air, in the presence of the pests on host plants. No claim is made for originality in any of the principles employed in the apparatus.

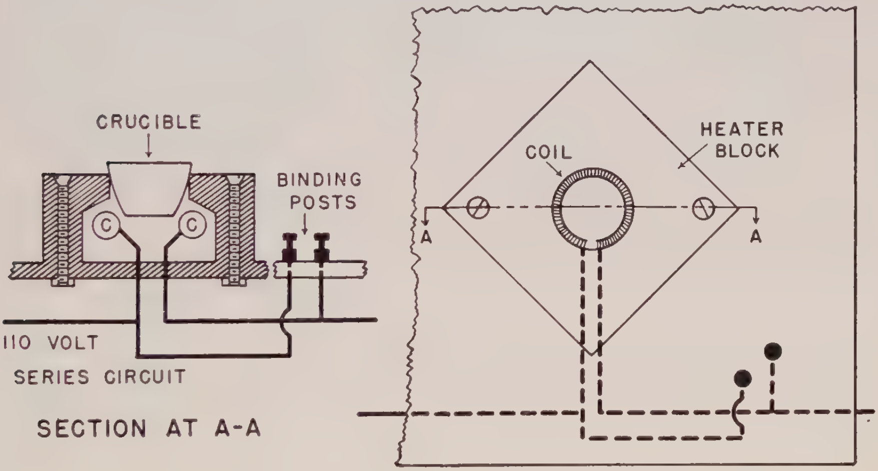

Figure 1. A single unit of the fumigator series.

A diagram of one of the units of the apparatus is shown in Figure 1. A photograph of a part of the series is shown in Figure 2. A small block of Transite, 1/2 inch in thickness, is fastened to a 14-inch square of Transite. A hole is drilled in the small block, diameter 1/2 inch at the top and 1 inch at the bottom. The coil is contained in the larger part of the hole. The opening in the top of the block will take porcelain crucibles of 18 millimeter outside top diameter.

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[1] The B. F. Goodrich Biochemical Laboratory at Boyce Thompson Institute for Plant Research, Inc., Yonkers 3, New York.

Although the number of units in one of these series is optional, the apparatus described has 10 units wired in series. Each coil has a resistance of 5 ohms, which would permit a current flow of a little over 2 amperes on a 110-volt circuit. This is sufficient to vaporize in 10 minutes or less any chemical that could be used as a fumigant. It has been standard procedure with this apparatus to run the current for just 10 minutes. It is important that the gauge of the resistance wire be sufficient to prevent continual burning-out. Nichrome wire, gauge 25, is satisfactory. This has a resistance of 2.1 ohms per foot.

In Figure 1 it may be seen that a by-pass circuit has been built into each unit. If, in the course of an experiment, a coil burns out, it may be located easily by means of a meter (voltmeter of 120 volts capacity) or a small 110-volt light bulb. The defective unit can be shorted out, and the test completed with the loss of one sample only.

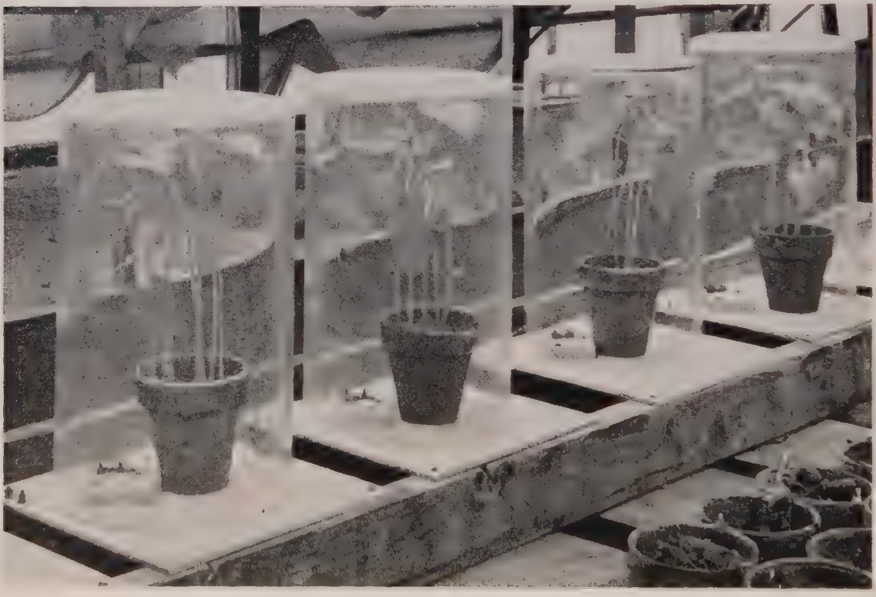

FIGURE 2. A few units of the fumigator series arranged for a test.

A 15-liter battery jar is satisfactory as a fumigating chamber. If desired, the jar may be sealed to the base with modeling clay. It is advisable to work a little glycerin into the clay to make it spread more easily.

It has been the practice to assay chemicals at an initial rate of 0.1 g. or, for liquids, 0.1 cc. in this apparatus. Chemicals effective at this concentration are then retested at lower concentrations. (There is evidence that in larger air volumes a dosage rate proportionately smaller will be effective.)

The fumigations are set up late in the afternoon, and the insects examined for kill the first thing the following morning. Sunlight is apt to increase injury to plants by the chemicals if the tests are set up earlier in the day.

With a ten-unit series, one operator can weigh out samples, set up the test, read out the test, and burn out the crucible residue with chlorate in not over two hours. One ten-unit series can thus handle forty or fifty samples a week.

APPARATUS USED IN LABORATORY INSECTICIDE TESTS AT BOYCE THOMPSON INSTITUTE

Albert Hartzell, Harry L. Haynes,[1] and Donald P. Connola[2]

This paper will describe the apparatus used in testing insecticides at Boyce Thompson Institute.[3]

Prior to the construction of the insecticide laboratory a number of other laboratories were visited with the idea of incorporating into plans of the new laboratory some of the most recent test apparatus. The writers are especially indebted to Dr. W. C. O'Kane, Durham, New Hampshire, for suggestions in constructing and equipping this laboratory, and to Dr. Harold A. Waters, Columbus, Ohio, for suggestions on rearing insects under artificial light.

The present discussion deals with some possible innovations of insecticide apparatus including a description of equipment used for culturing insects under controlled conditions of temperature, humidity, and light. A later paper will describe the methods employed using this equipment.

No attempt will be made here toward an historical review of insecticide testing apparatus in general. The following citations have come to the attention of the writers in the preparation of this article. In 1923, Tattersfield and Morris (14) described an apparatus for testing the toxic values of contact insecticides under controlled conditions. It was designed on the assumption that if successive numbers of similar insects are placed upon a plane surface in a given area and sprayed uniformly under constant conditions as to the amount of liquid, pressure, spreading, wetting and adhering properties at the same concentration of toxic substance, the amount of poison received by each insect will be equal. The apparatus consisted of a glass jar with a wooden cover and a means of leveling both the cover and a small open dish inside the jar containing the insects to be sprayed. There were arrangements for supplying air at a constant known pressure and for holding and adjusting the spray nozzle. With this apparatus Tattersfield and Morris (14) on the advice of R. A. Fisher demonstrated conclusively for the first time that concentrations which give kills of approximately 50 per cent of the insects sprayed are the most reliable for the direct comparison of the toxicities of insecticides. O'Kane *et al.* (8) in 1930 described

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[2] Formerly a Crop Protection Institute Fellow.

[3] This equipment has been made available under a cooperative arrangement with Carbide and Carbon Chemicals Corporation and Crop Protection Institute.

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a turntable for applying insecticidal sprays. A phonograph motor with multiple spring and automatic adjustable governor was used to revolve the turntable at speeds ranging from 15 to 150 revolutions per minute. In fungicide tests McCallan (4) used a turntable driven by an electric motor. The turntable was mounted in a spray hood. Peterson (11, 12), in his manual of entomological equipment and methods, has assembled from widely scattered sources drawings and descriptions of many methods used in experimental entomology. Waters (15, 16, 17) has been unusually successful and ingenious in rearing insects under artificial conditions, as pointed out by Osborn (9). Spray chemicals and application equipment are described and illustrated by McClintock and Fisher (7) in their recently published textbook. Some of the more modern types of power-driven spraying and dusting equipment are described and illustrated. Shepard (13) has discussed biological methods of testing insecticides. These methods he states may be used in discovering new materials in screening operations, to develop new uses in formulations for practical application, to make fundamental studies of toxicity and the mode of action, to provide control in commercial production and government regulation, and to supplement chemical analyses with biological assays. A direct spray technique for the biological evaluation of pyrethrum-in-oil insecticides against stored products insects has been described recently by Hewlett (3).

GENERAL DESCRIPTION OF LABORATORIES

Two rooms were constructed, one (30 ft. × 24 ft.) for culturing insects under controlled temperature, humidity, and light (Fig. 1 B), the other a testing laboratory (Fig. 1 A) 24 ft. square for spraying, dusting, and fumigation. In addition, a small adjacent room (8 ft. × 8 ft.) was built for rearing household and stored products insects not requiring light.

CULTURE ROOM FOR INSECTS

The room for culturing insects under artificial light previously referred to is maintained at 80° ± 1° F., and a relative humidity of 50 per cent by means of a Carrier air-conditioning unit. The air is changed once each 12 minutes. As this is a dewpoint system of air-conditioning it is dependent on the temperature of the tapwater remaining below 60° F. During the summer months the water before entering the humidifier is cooled by means of a York Freon cooling unit. Each of the four light cages (71 in. × 56 in., height 36 in.) is illuminated by two units each containing two 100-watt mercury vapor lamps 38 inches long. The cages are fitted with stainless steel trays that can be drained conveniently. The sides of the cages are covered with stainless steel 28 mesh and can be removed if necessary. There are doors for introducing insects.

Cultures of Mexican bean beetle (*Epilachna varivestis* Muls.), Southern

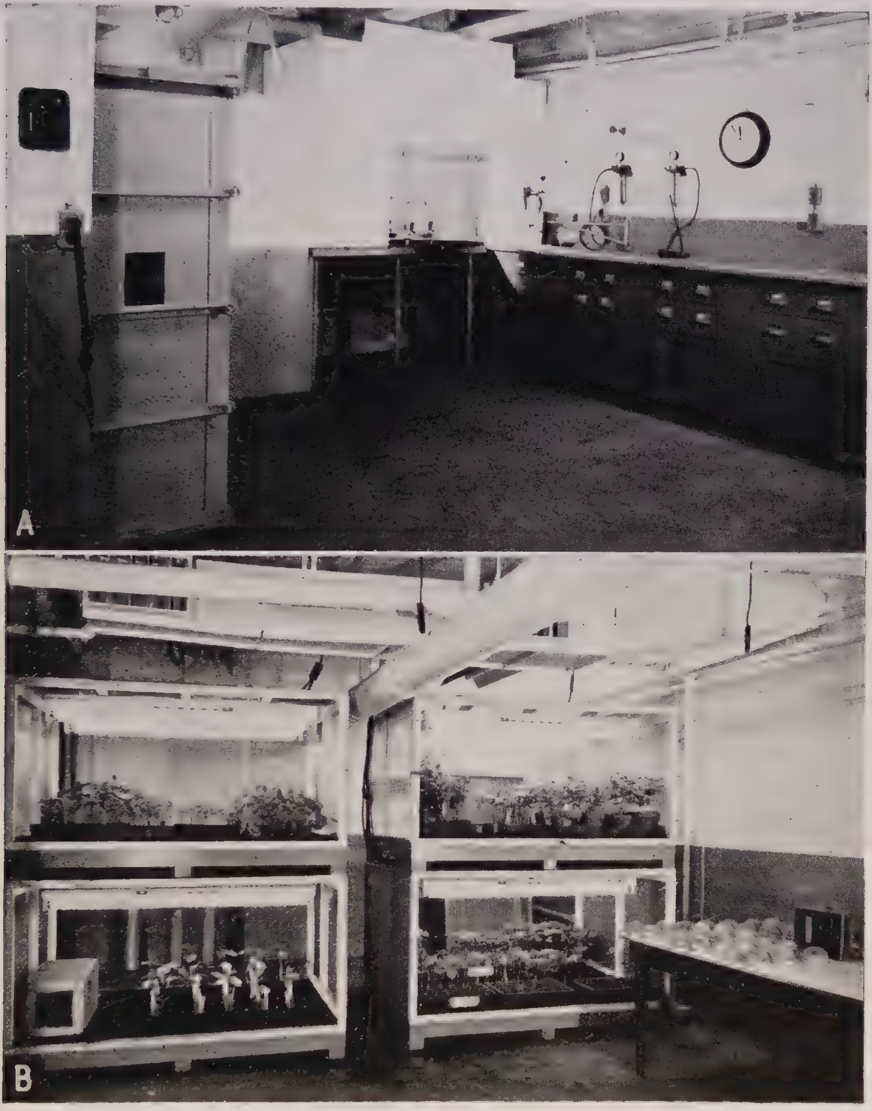

FIGURE 1. A. Insecticide testing laboratory showing spray and dust hood with motor driven turntable. Fumigation chamber at the left. B. Room for culturing insects under controlled conditions of temperature, humidity, and light, showing fluorescent light cages.

army worm (*Prodenia eridania* Cram.), black bean aphid (*Aphis rumicis* L.), pea aphid (*Macrosiphum pisi* Kltb.), and mite species (*Tetranychus bimaculatus* Harvey and *Tetranychus althaea* McGregor) have been reared successfully under fluorescent light.

The following were grown for the foliage-chewing insects: pole bean

(*Phaseolus vulgaris* L.), bush bean (*P. vulgaris* L. var. *humilis* Alef. "Tendergreen" and "Cranberry"). Nasturtium (*Tropaeolum* sp.) plants, dwarf and climbing varieties, are used for rearing bean aphids, and pea aphids are reared on Windsor bean. Different species of mites, depending on the host plant, have been cultured very successfully on Tendergreen bean, cotton (*Gossypium hirsutum* L.), and castor bean (*Ricinus communis* L.) plants. Both in the greenhouse and in the culture room mites do much more poorly on Cranberry beans than on Tendergreen beans.

All the plants mentioned above have been grown under fluorescent light although the growth is somewhat retarded. The plants become spindly and the broad leaves do not develop normally. Under our conditions the plants are grown in the greenhouse and then transferred to the fluorescent light cages for the insect cultures as it is difficult to grow plants for their entire growth period in artificial light (1). Growth is slightly retarded when the plants are moved to the artificial conditions. In our initial studies 14 hours of fluorescent light were found to be required for maximum growth of the plants.

Peat moss soaked with water has been used in the metal trays in preference to standing water. The dampened moss furnishes a large evaporating surface and helps to control the humidity in the cages. For example, the peat moss in a cage containing red spider mites is kept much drier than in a cage where Mexican bean beetles are being reared.

The fluorescent light cages and other available space in the culture room are used also for holding tests under constant conditions of temperature, humidity, and light.

TESTING LABORATORY

Spray and dust hood. In the testing laboratory the combined hood (Fig. 1 A) for spraying and dusting is constructed of Flexboard with an alberene stone floor. The hood is 38 inches in length, 36 inches deep, with an average height of 52 inches. The air exhaust is of an average velocity of 40 feet per minute through an opening 36 inches by 29 inches, or 290 cubic feet of air per minute. The opening of the exhaust pipe is provided with a damper. The hood has a sliding window (2-1/2 ft.×3 ft.) in front through which the spray is introduced. When the hood is used for dusting the window is closed and the dust is introduced through a similar window in the side of the hood. Baffles prevent the dust from escaping into the laboratory.

Spray apparatus and turntable. The spray apparatus, as illustrated in Figure 1 A and Figure 2 A, is similar to the type described by McCallan and Wellman (5) except for several new adaptations. The DeVilbiss

(CH 6 2041) spray gun is mounted on a swivel arm which is attached to a galvanized iron pipe fastened to the wall. The "paint" spray gun can be rotated on a horizontal axis of 270°, and 180° on a vertical axis. The spray arm has an overall length of 39 inches which includes a telescopic extension of 15 inches. The gun has a variable height of 14-1/2 inches. The spray gun accordingly can be adjusted to operate from many fixed positions facing the hood and at many different angles and heights. This adds to the versatility of the apparatus as it can be utilized for several different operations.

The air pressure for the spray gun is supplied by the air-reducing valve, DeVilbiss type HB 302, as shown in Figure 2 A (left). Another air-reducing valve (right) is available for the air brush (Wold No. 631) which is used for the fly and cockroach chambers.

Cockroach chamber. As the dose of a fly spray ordinarily has to be doubled to kill cockroaches, considerable difficulty was experienced in scrubbing the Peet-Grady chamber after tests on cockroaches to remove spray residues because of the large surface involved. In order to avoid this, a cockroach chamber (Fig. 3 A and B) made of a B29 bomber nose was mounted on an iron stand 33 inches high. A plate glass circle 38 inches in diameter was mounted on a carriage that could be carefully slid under the stand. The carriage was provided with a ratchet that lifts the glass discs to meet strips of plate glass cut to fit snugly the disc on the carriage. A removable Plexiglas door approximately 18 inches in diameter provided with turn-buckles permits the introduction of the cockroaches in Petri dish cages into the chamber. The roaches are placed in Petri dish cages covered with 18 mesh wire. The cages are placed in the center of the plate glass disc. After spraying from the top with an air brush, the cockroaches are removed from the chamber and held at Peet-Grady rearing conditions for observation. The chamber and disc are scrubbed with alcohol to remove residue before another test is conducted. The chamber has a capacity of 16.5 cubic feet, or approximately 1/13th that of a standard Peet-Grady chamber.

Fumigation chamber. The fumigation chamber (28-1/2 in. × 23 in., height 70 in.) is illustrated in Figure 3 C. It is constructed of Flexboard and has stainless steel supports. The chamber is equipped with a hinged door containing a glass window (8 in. × 6 in.), two 8-1/2 inch exhaust pipes, a removable 1/2 inch mesh screen shelf, and electrical outlets. Vapors are removed in a few seconds through the same exhaust system that evacuates the hood by opening the top exhaust pipe followed by opening the bottom valve which allows fresh air to enter. By this operation all harmful vapors can be removed before the door of the chamber is opened. Non-sparking electrical outlets controlled from the outside supply the chamber with light

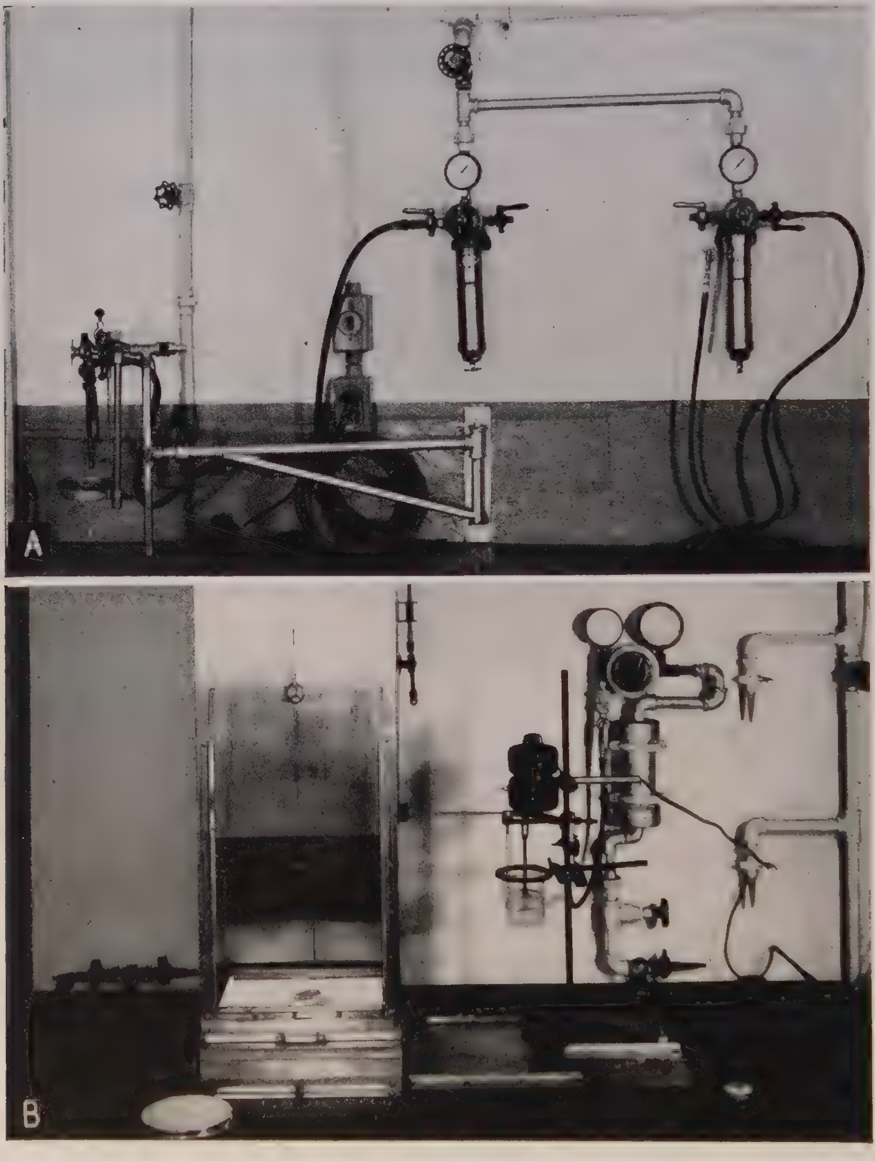

FIGURE 2. A. Telescopic arm of spray gun retracted. Air reducing valves, left for spray gun, right for air brush for cockroach and fly spray tests. B. McCallan and Wilcoxon spray settling tower. Modified for aphid tests in Petri dishes.

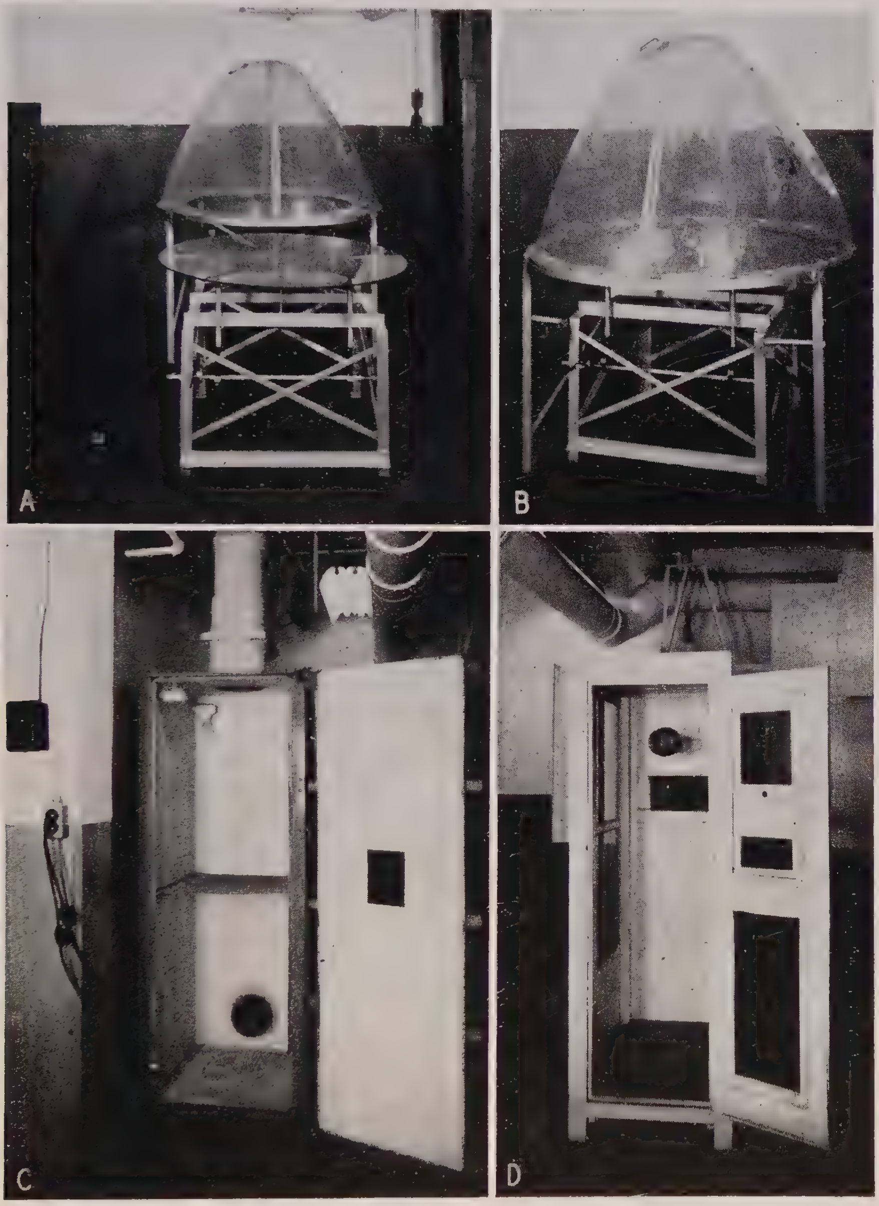

FIGURE 3. A. Cockroach spray chamber with removable plate glass disc bottom mounted on carrier. B. In position for spraying cockroaches. Note small cages in center of disc. C. Fumigation chamber showing exhaust pipe and cutoff. D. Fly knockdown and kill chamber.

and provide for the use of apparatus within the closed chamber such as a hot plate or fan. Liquids or gases can be introduced through a small circular aperture on the right-hand side of the chamber.

Fly knockdown and kill chamber. A smaller piece of apparatus than a Peet-Grady chamber was desired in which more compounds could be screened per day on flies and whereby these results would be analogous to Peet-Grady tests. The chamber designed for this use is illustrated in Figure 3 D. It consists of a wood and glass chamber 68 inches high and 24 inches wide with a depth of 36 inches. The spray is applied through a small orifice at the front of the cage. To insure thorough distribution of the mist a permanently mounted fan on the back of the cage blows an air stream through a 30 mesh screen directly at the incoming spray. The fan is turned off immediately after the spray is introduced and the cage is completely closed by inserting a tight fitting panel in front of the fan. Observations are made only on the number of flies that are knocked down. After the exposure period all active and immobilized insects are removed from the chamber by a vacuum cleaner type suction apparatus.

To measure the toxicity of the chemicals, flies are enclosed in an 18 mesh wire spherical cage, 5 inches in diameter, which is suspended in the chamber and exposed to the spray mist for the same period of time as the other batch of flies tested for knockdown. The spherical cage is hung just above the direct line of the spray so the flies or cage are not hit directly by the spray. Immediately after the exposure period the caged flies are transferred to an observation cage and stored according to Peet-Grady specifications.

SETTLING TOWER

The settling tower developed and described by McCallan and Wilcoxon for the testing of fungicides (6) is illustrated in Figure 2 B. This settling tower has been used very efficiently for screening compounds on aphids. Detached leaves of plants infested with aphids are placed in Petri dishes in the bottom of the tower. Then the spray is introduced into the tower, the spray orifice being directed upwards, and the insects are exposed to the settling mist for a given period of time. The dosage is controlled by the amount of spray being introduced in the tower and the time the insects are subjected to the settling droplets.

OTHER APPARATUS

In addition to the apparatus described in the foregoing section of this paper several methods utilizing standard equipment are in use at the Institute. For the past eight years the Institute has maintained a housefly spray testing laboratory using the large chamber Peet-Grady method (10).

In addition, apparatus for housefly repellency, stored products insects, and mosquito larvicide (2) tests are available. As these methods employ standard equipment, it is not necessary to describe them here.

SUMMARY

Apparatus used in conducting insecticide tests at Boyce Thompson Institute is described and illustrated.

It consists of a series of cages in a culture room for rearing insects under controlled conditions of temperature, humidity, and light, and a testing laboratory containing a combined spray and dust hood with a motor driven turntable mounted in it. Spray apparatus, cockroach, fumigation, and fly knockdown chambers located in the same laboratory also are described.

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SUGGESTED METHODS FOR MEASURING THE CARBON DIOXIDE PRODUCTION OF A LARGE NUMBER OF SIMULTANEOUS SAMPLES

F. E. Denny

The method perhaps most commonly employed in measuring the carbon dioxide production of plant tissue consists in drawing a current of air through the container which holds the sample, and absorbing the eliminated carbon dioxide in a solution of alkali.

This method, however, is hardly feasible if there are a large number of samples to be tested simultaneously, and especially if the duration of the experiment requires an over-night run for the apparatus. Under these conditions the equipment required for maintaining a continuous flow of such large volumes of air would have to be quite sturdy and of large capacity; also both the machinery and the gas-absorbing system would need to be of exceptional dependability if the services of an attendant during the night are to be dispensed with.

The need of a method for a large number of simultaneous samples became apparent in a study (soon to be reported upon in the Contributions) of the relation of the respiration of a tissue to the effectiveness of various chemicals in retarding the growth of the surface micro-flora. Even if a test involved only two chemicals, each at three concentrations, triplicate sampling would require 18 simultaneously-running lots.

Two different methods of dealing with such a situation were used. Since it appeared that these methods might have general application in experiments relating to carbon dioxide production when a large number of simultaneous samples were required, the methods are described separately from the data derived from the experiment in which they were developed. They may prove useful whenever several replicates of each of a number of treatments are required, or when an experiment of the factorial type is encountered, in which several factors and their interactions need to be studied in a single test.

METHOD I

OXYGEN CONSUMED REPLACED BY OXYGEN

The arrangement of the apparatus to furnish a continuous supply of oxygen to the tissue is shown in Figure 1 A and B. At the end of the capillary tube which extends through the stopper of the fruit jar (Fig. 1 A) a

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t-tube is connected; a slow current of oxygen is caused to pass continuously through this *t*-tube; as oxygen is consumed by the respiration of the tissue, the carbon dioxide formed is absorbed by the layer of alkali in the bottom of the jar (not shown in the figure because of interference of the moisture vapor in making the photograph), and oxygen is brought into the jar from the *t*-tube through the capillary.

By connecting the *t*-tubes of a number of these jars in series the oxygen supply can be made to suffice for all of the jars. Thus, in Figure 1 B is shown a set-up of 36 jars operating simultaneously. The 19-liter bottle at the left contains the oxygen obtained from a cylinder of the compressed gas collected over water. The five gallon pail on top of the rack contains water which is siphoned over, drop by drop, into the funnel by means of the long capillary tube, the oxygen gas being pushed continuously through the entire system of connecting *t*-tubes. The rate of dropping is regulated by the bore and length of the capillary tube leading from the water reservoir. In this particular case the rate was about 19 liters in 24 hours. If the rate of respiration in the jars is very high two such oxygen supply bottles may be put in operation, and the tubing connection changed to the second bottle at the half-way place on the rack. Presumably, instead of using the oxygen bottle, the tubing could be connected directly to a cylinder of compressed gas equipped with a reducing valve, but this was not done in these tests. For the preparation of the glass stands in the bottom of the jars, and the screens on which the tissue is placed, reference is made to a previous article (3).

In actual operation the front and rear of the rack carrying the jars were draped with a curtain made from four thicknesses of cheesecloth. This was to reduce the force of the air currents from the automatic refrigeration apparatus which was of the on-and-off type. A thermograph placed on the rack between a pair of such screens showed a nearly straight temperature line with only very small waves due to the intermittent exposure to cold air from the refrigeration unit.

The carbon dioxide produced by the sample of tissue was absorbed in a layer of alkali pipetted into the bottom of the jar at the start of the test, the amount depending upon the expected rate of respiration and usually being 25 or 50 cc. of 2 N NaOH. At the end of a test this liquid was rinsed into a 500 cc. volumetric flask; a quantity of saturated $BaCl_2$ solution sufficient to effect complete precipitation was added; the flask was then made up to the mark, and after mixing and allowing the precipitate to settle, aliquots were taken for titration against 0.5 N or 0.25 N HCl with phenolphthalein as indicator.

A large number of tests were made with this method in connection with experiments (to be reported upon in a succeeding issue of the CONTRIBUTIONS) relating to the effect upon the respiration of seeds resulting from

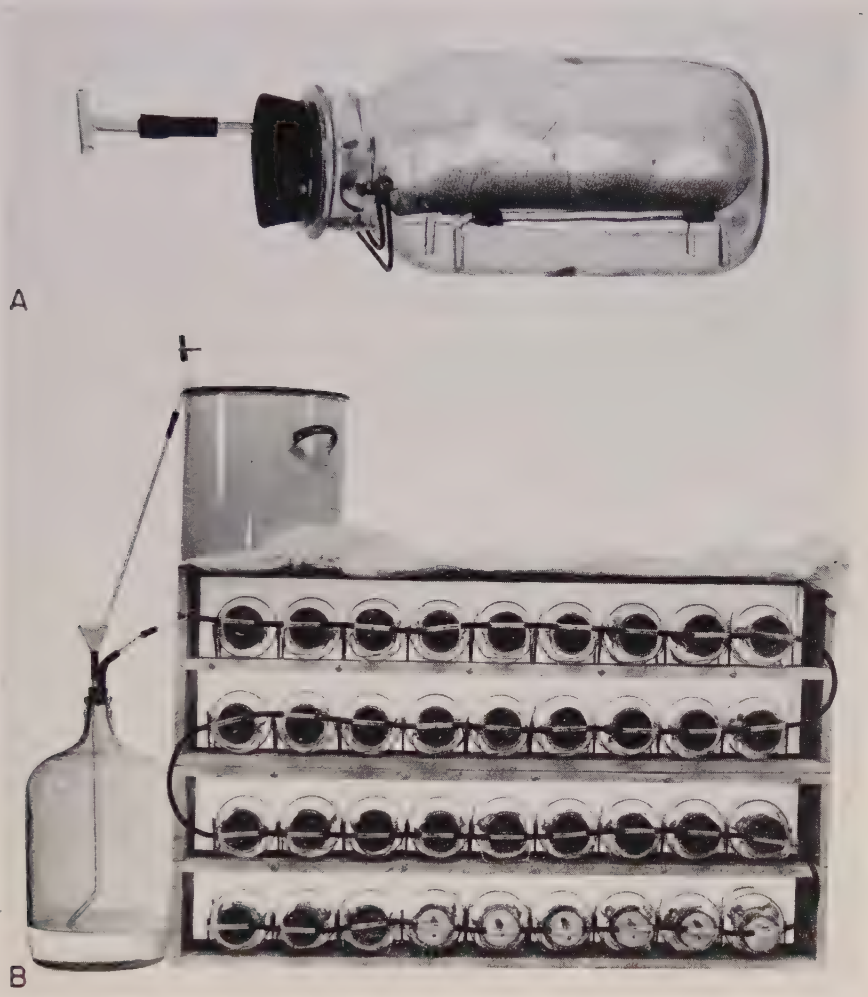

FIGURE 1. A and B, apparatus for Method I, supplying oxygen to respiring tissues as fast as it is consumed in respiration. A. Previously soaked seeds in cheesecloth bags on screen supported by shelf; a layer of 2 N NaOH (not shown in the photo) absorbs CO_2 produced. A very slow current of O_2 is made to pass continuously through the *t*-tube; decrease in pressure in jar due to utilization of O_2 draws in an equal volume of O_2 through the capillary tube connecting the *t*-tube and the inside of the jar. B. A set-up of 36 respiration vessels similar to that shown in A, the *t*-tubes being connected in series; the 19-liter bottle at the left contains oxygen gas; this is displaced by water siphoning over drop by drop from the 5 gallon pail through the long capillary tube leading to the funnel; the length and bore of the capillary regulates the rate of dropping; the slow current of O_2 traverses the entire circuit of tubing and is discharged into the air at the last jar (lower left). The *y*-tube at the top of the siphon provides for the collection of air bubbles that might be formed in the siphon and stop the slow flow of water.

the use of chemicals in retarding the growth or possibly other activities of the surface micro-flora of the seeds. Since several chemicals, different concentrations of each chemical, and four to six replications of each treatment were involved, the need of a method for many simultaneous samples became apparent. This method gave satisfactory results under such conditions, as will appear in the published article.

A few tests were made to compare Method I with the usual aeration method in which the carbon dioxide was swept out of the jars in a current of air and was absorbed in alkali as fast as it was eliminated. The apparatus used for this purpose was that originally devised by Miller (5, 6), a solution of NaOH being used as the absorber rather than one of the $Ba(OH)_2$. Wheat (*Triticum aestivum* L., Leap's Prolific) seeds which had been soaked for 24 hours before being placed in the respirometers were used. The seeds were tied in cheesecloth bags, 8 bags in each lot, 8 g. of air dry seeds (presoak weight) in each bag. The experiments each ran for 6 hours and 20 minutes. The results from two such experiments are shown in Table I. Although the results are slightly higher by the aeration method the analysis of variance does not show a method effect at the 0.05 level of probability. The over-all difference is about 2.5 per cent and more extensive tests would be required to decide the point. However, it is not regarded that these two

TABLE I

COMPARISON OF RESULTS BY METHOD I AND BY THE AERATION METHOD

Cc. of carbon dioxide produced			
Experiment I		Experiment II	
Aeration method	Method I	Aeration method	Method I
89	87	92	85
82	89	86	89
93	89	95	90
89	89	90	93
95	81	98	90
81	88	97	90
529	523	558	537

Analysis of variance

	D.F.	S.S.	Var.
Total	23	464	—
Exp.	1	77	77
Method	1	30	30
M×E	1	10	10
Repl.	20	347	17.35

methods are competitive. Method I is intended for large numbers of simultaneous samples under which conditions the aeration method can hardly be considered feasible. The present results indicate that if the results by the aeration method be taken as correct, the results by Method I are not seriously in error.

Furthermore, we need not assume that the aeration method is necessarily correct, for it has its own objectionable features. In the first place, the tissue is subjected to a continuous negative pressure of about 2.0 centimeters of mercury, and secondly, the flowing of a rapid current of air around the tissue represents, at least in the present case, a particularly unnatural set of conditions. The effect of such factors on respiration rate is not known.

Although the apparatus keeps available to the respiration jars a constant supply of oxygen to replace that consumed inside the jars, it is not necessarily true that there is no change in the proportion of oxygen within the jars during the test interval. Gas analyses of the air inside the jars at the end of a test usually showed somewhat more than 20.9 per cent oxygen, increases of one or two percentage points being found. The oxygen content inside the jars is influenced by changes in temperature, barometric pressure, and by the respiratory volume ratio, $CO_2 \div O_2$. The effect of the first of these can be eliminated by control of temperature (which was done in these tests); but the second and third of these difficulties cannot be avoided. An estimate of their effects can, however, be made. With Method I, a *fall* in barometric pressure has no effect upon the volume percentage of oxygen inside the container: the gas which is present merely expands but the percentage composition remains the same. However, if the barometric pressure *increases*, then some pure oxygen is pressed into the container and the percentage of oxygen is increased. Under ordinary conditions this barometric effect is not large. If the barometric pressure increased by 10 mm. during an experimental interval, the increase in the percentage of oxygen would be about $(100 \times 10) \div 760 = 1.3$.

The respiratory ratio, $CO_2 \div O_2$, has no effect upon the percentage of oxygen in the container if the ratio is 1.0 or greater; the excess of CO_2 is not accompanied by a corresponding intake of O_2, and the absorption of this extra CO_2 does not cause a reduction in pressure in the container, and so no O_2 is drawn into the container because of its absorption. See (1, p. 390) for a discussion on this point. When, however, the respiratory ratio is less than 1.0, an excess of O_2 is drawn in beyond that caused by the formation of CO_2 resulting from a reaction involving atmospheric O_2, and the percentage of O_2 in the container is increased. Thus if the ratio, $CO_2 \div O_2$, were 0.87, for each 100 cc. of CO_2 produced, 115 cc. of O_2 would be consumed, and the excess O_2 taken in beyond that involved in producing CO_2 directly would be 15 cc. of O_2. If the net air volume of the container were 1500 cc. the increase in the percentage of O_2 in the container would be 1.0.

Of course, both of these factors might be operating simultaneously which would cause an increase of about 2.3 in the volume percentage of O_2 within the jar.

This brings up the question whether changes in the O_2 concentration within the range from 20.9 per cent to, say, 24 per cent affect the respiration rate. Published data for this range were not found. Values by Mack (4, p. 44) at 20° C. showed an increase of about 10 per cent in the respiration rate by changing the O_2 concentration from 20 per cent O_2 to 30 per cent O_2. Steward (7, p. 220) shows a curve covering a wide range of O_2 concentrations, and by interpolation on his graph we may estimate an increase of about five per cent in the respiration rate by a change in O_2 concentration from 20.9 per cent O_2 to 30 per cent O_2. The effect of O_2 changes in this range appear to be small, and since the supposed conditions in this discussion are rather extreme, i.e. a drop of 10 mm. in the barometer and a respiratory ratio of 0.87, it seems reasonable to believe that under most ordinary conditions the respiration rate will not be influenced importantly by any small increases which may occur in the O_2 concentrations in the containers.

METHOD II

OXYGEN CONSUMED REPLACED BY AIR

Some tests were made to note what would occur if the apparatus for supplying oxygen in Method I was detached, and air rather than oxygen was allowed to enter the container to replace the oxygen consumed. The apparatus then appears as shown in Figure 2. It consists merely of a respiration chamber connected to the outside air by a short capillary tube. As oxygen is used up in the container a volume of air needed to equalize the reduced pressure enters. Since the entering air contains only 20.9 per cent O_2 there is a gradual depletion of the oxygen in the jar. But it was shown in a previous paper (2) that, at least for the tissues dealt with in that experiment, a depletion of the O_2 from air content down to about 16 per cent O_2 did not materially affect the rate of respiration.

A few tests were made comparing the three methods, i.e. continuous aeration, Method I, and Method II. Soaked wheat seeds were used and each experiment ran 6 hours and 20 minutes. The results are shown in Table II. Again the aeration method may be slightly higher, but the analysis of variance does not show a significant treatment effect at the 0.05 level.

Because of the simplicity of Method II, it deserves further testing. It seems particularly well-suited for occasional or preliminary tests, or for an

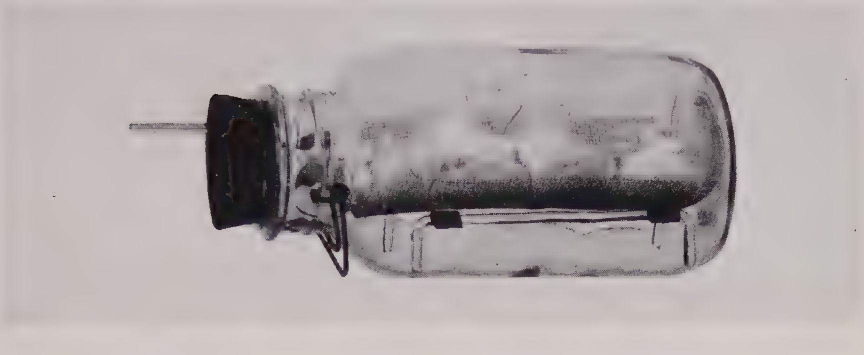

FIGURE 2. Apparatus for Method II, air instead of O_2 being supplied to the tissue as fast as O_2 is consumed. The CO_2 formed is absorbed in a layer of 2 *N* NaOH in the bottom of the jar (not shown in the photo) and the resulting decrease in pressure draws air in through the glass capillary tube leading to the outside air. Since the incoming air has an O_2 content of only approximately 20.9 per cent there is a gradual depletion of the O_2 in the container. By a suitable choice of the volume of the container and the size of the sample of tissue it can be assured that the final O_2 concentration shall not fall below a specified value. See text for a formula relating the three variables.

TABLE II

COMPARISON OF RESULTS BY THE THREE METHODS

Exp. No.	Cc. of carbon dioxide produced		
	Aeration method	Method I	Method II
I	51	57	56
	63	56	58
	64	57	54
	61	51	58
II	58	61	60
	63	63	67
	63	60	58
	68	63	63
Totals	491	468	474

Analysis of variance

	D.F.	S.S.	Var.
Total	23	447	—
Exp.	1	155	155
Method	2	35.6	17.8
M×E	2	11.15	5.58
Repl.	18	245.25	13.62

orientation experiment designed to determine the critical phases of a problem that need closer study.

To make use of Method II, it is necessary to know, at least roughly, about what the respiration rate is to be, so that the size of the sample and the volume of the container can be so adjusted that the percentage of O_2 in the container at the end of a test will be not lower than some designated amount, say, 18 per cent, 16 per cent, etc. This is to avoid very low oxygen percentages which would materially retard the rate.

If the approximate rate of respiration of the tissue is known or has been determined at least roughly by a preliminary test (and Method II itself would be convenient for this), a formula may be used for determining the proper volume of the container to use, and the amount of tissue to take in the sample, so as to insure that the final percentages of oxygen in the container shall not fall below a certain specified value.

The derivation of the formula relating these three variables to each other is as follows. In a vessel of volume V (i.e. net air volume after deducting the volume of the sample, solution, shelf, etc.), if r cc. of O_2 are consumed (on account of which r cc. of air are drawn into the jar), and if it is desired that the final O_2 proportion by volume shall not fall below a specified value, say c, i.e. 0.18, 0.16, etc., we have the following:

cc. O_2 in container at start $= 0.209\ V$
cc. O_2 consumed during test $= r$
cc. O_2 drawn in with entering air $= 0.209\ r$
cc. O_2 in jar at end $= 0.209\ V + 0.209\ r - r$

$$\text{Volume proportion of } O_2 \text{ at end} = \frac{0.209\ V + 0.209\ r - r}{V}$$

If it is desired that this proportion be equal to, say c;

$$\text{Then, } \frac{0.209\ V + 0.209\ r - r}{V} = c$$

$$0.209\ V + 0.209\ r - r = cV$$
$$0.209\ V - cV = r - 0.209\ r$$
$$(0.209 - c)\ V = (1 - 0.209)r$$

The value of the factor in front of r is, of course, 0.791, but it seems better to leave the equation in that form to show the derivation of the value.

After any two of these variables have been fixed the third may be estimated. For example, if it is desired that the final percentage of O_2 shall not fall below 17 per cent, and if it is desired to take a sample sufficiently large to obtain a reading of 100 cc.:

$$(0.209 - 0.17)\ V = (1 - 0.209)100$$
$$V = \text{approx. 2 liters}$$

As another example: if the net air content of a container is 3000 cc.; and if the volume proportion of O_2 is not to fall below 0.18, the sample taken should be one that will not produce more than about 110 cc. of CO_2 in the respiration period.

These values are of course only approximate. Ordinarily r must be within reasonable limits in order that a dependable reading can be made. It will be noted that a small change in c causes a relatively large change in V.

TEMPERATURE EFFECTS UPON THE OXYGEN CONCENTRATION IN THE CONTAINERS

Although the present experiments were carried out in a constant temperature room (at 20° C.), it is not essential for the success of either method that the temperature remain closely constant. Change in temperature will have some effect upon the concentration of oxygen in the container but this is not an important factor for reasonably small changes in temperature as will be seen from the following considerations.

In Method I an *increase* in temperature produces no change in the percentage of O_2 in the container. The gas already present expands but the volume relationship of the gases does not change. If the temperature *falls*, however, some oxygen is drawn into the container from the oxygen supply, and the volume percentage of O_2 increases. The relation between the decrease in temperature and the percentage of O_2 in the container may be derived as follows:

If V is the volume of the container, the O_2 present at the start $= 0.209\ V$

If the temperature falls by t degrees C. the volume decreases by $\frac{t}{273}\ V$

And the amount of O_2 drawn into the container is $\frac{t}{273}\ V$

The new volume of O_2 in the container is now

$$0.209\ V + \frac{tV}{273} = \left(0.209 + \frac{t}{273}\right) V$$

And the volume percentage of

$$O_2 = \frac{\left(0.209 + \frac{t}{273}\right) V}{V} = \left(0.209 + \frac{t}{273}\right) = \frac{57+t}{273}$$

If we designate c as the final volume concentration of O_2

$$c = \frac{57+t}{273}$$

Knowing one of these values the other can be computed. If the temperature falls 3° C. the volume percentage of O_2 will be $\frac{57+3}{273}=0.22$. How many degrees decrease in temperature will be required to increase the oxygen concentration to 24 per cent?

$$.24=\frac{57+t}{273}$$

$$t=8.5° \text{ C.}$$

It, thus, appears that a considerable fall in temperature is required to change the volume percentage of oxygen to the point at which it seems possible that an effect upon the respiration would result. There would be a large direct effect of the temperature on the respiration, but a much smaller and usually an insignificant effect on respiration due to the indirect effect of change in oxygen concentration on respiration rate.

Method II is also affected by a change in temperature. In this case, *decrease* in temperature has a favorable effect so far as the O_2 concentration in the container is concerned, since some oxygen is drawn in with the air, and this tends to delay the time at which the O_2 becomes depleted to whatever value is set as a lower limit. An *increase* in temperature causes loss of internal gas but this gas ordinarily will contain less than 20.9 per cent by volume of oxygen, so that the amount of O_2 becoming unavailable for further respiration on account of temperature increase will usually be small.

Temperature effects can be minimized by using basement or other rooms with only gradual changes in temperature, and further by immersing the respiration vessels in large volumes of water. If such containers as those shown in Figures 1 and 2 are placed in water a bent-tube adapter can be connected to the capillary tube extending through the stopper of the respiration vessel.

SUMMARY

The method commonly employed to measure the carbon dioxide production of plant tissue is unsuitable for a large number of simultaneously-run samples. Two different methods are suggested for this purpose. With one, the oxygen consumed is replaced continuously by an equal volume of oxygen, while with the other the oxygen consumed is replaced by air. In the latter case the oxygen supply available to the tissue is progressively partially depleted, but it is shown that by properly choosing the size of the container and the sample, oxygen concentrations unfavorable for maintaining respiration rate may be avoided. A set-up of apparatus by means of which 36 samples may be run simultaneously is described. The pieces of apparatus used are such as are usually available from an ordinary stockroom supply. Control of temperature is desirable, but close regulation is not essential, much valuable information being obtainable without it.

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A METHOD FOR CORRECTING FOR GEOGRAPHIC VARIATION IN FIELD EXPERIMENTS[1]

R. H. Wellman, H. W. Thurston, Jr., and F. R. Whaley[2]

INTRODUCTION

The purpose of much field experimentation is to compare varieties or treatments as precisely as possible. In the past, uniformity trials have been used frequently to determine yields independent of any intentionally imposed variables. Such trials are finding less use currently because of their time-consuming nature. Further, although they indicated homogeneity, or lack of it, for a given set of conditions, they did not indicate how yields might be affected by different conditions. The information obtained in most field trials must be a composite of the variations obtained in uniformity trials and variety or treatment effects. The variations of uniformity trials are in turn divided into two parts: uncontrolled unorganized variation (the true error of an experiment) and an organized variation attributable to geographic position of each plot within the experiment. This latter variation can be caused by such factors as weediness, air drainage and direction of prevailing winds, as well as by soil fertility and moisture. Hence it would be improper to call it soil variation. In this paper the net result of this complex of factors will be termed geographic variation and the correction applied to minimize it will be called a geographic correction. The problem becomes one of separating as completely as possible geographic variation from all other factors involved and then, by correction, to minimize the geographic variation without disturbing either treatment effect or the true error variation.

The use of currently available designs, such as Latin squares and lattices severely restricts the number of replicates and treatments that can be used in an experiment. They make an indirect approach to minimizing the continuously changing geographic variation by dividing the area under test into smaller blocks. However, each block is treated as a discontinuous unit and no method is available to take into account the geographic variation within a block. Further, in certain designs, comparisons between treatments not occurring within the same block cannot be made with the

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same precision as that between treatments which do occur in the same block.

The purpose of this paper is to describe a correction that does take into account the effect of geographical position on yield. The method is empirical and a statistical background has not been developed for it. It is to be hoped that this will be done by someone more competent or that the method can be shown to be unsound. The authors have found the method very useful over a period of four years in interpreting potato yield data.

After the method had been worked out it was found that Richey (2, 3) had published two excellent articles on the same general subject. His articles were written before the effect of moving average corrections on reduction of degrees of freedom for error was fully appreciated. The method presented here lessens and under certain conditions avoids this difficulty.

The general procedure for this correction is outlined in the following section. The authors have found that the mechanics of the correction can be most readily understood by working the example that follows the general explanation. For those interested in attempting to apply the correction it is suggested that the general explanation be read first, then the example carefully followed, and finally the general section reread.

PROCEDURE FOR THE CORRECTION

The first equation necessary for this correction is:

$$\frac{\overline{Y}}{y} \times R = G. \tag{i}$$

Where R represents individual plot yield[3] (plot is defined as a single replicate of a single treatment) and $\overline{Y}$ equals the average yield per plot for the entire field. Let y equal the average yield per plot for a given treatment. By using formula (i) for each y value successively G values for each plot will be obtained. The G value is the expected plot yield independent of treatment. In other words, each G value is the most likely value that would have been obtained (for the plot whose y value went into the equation) if a uniform treatment had been applied, assuming further that such a uniform treatment would have given the same average yield ($\overline{Y}$) as that obtained in the experiment.

G values are calculated for each plot and are used to construct a graphical yield contour of the field (Fig. 1).

The abscissa values are the geographical position of the plots in the field and the ordinate values are yields (or other measurements). Adjacent plots are averaged in groups; in Figure 1 in groups of three. This is not a moving average. The first three are averaged, then the second three; not 1, 2, 3 and then 2, 3, 4. The use of groups of three is arbitrary. In other instances groups of four have been used in making averages. Since a smooth curve is drawn through these averages, it is felt that the degrees of freedom used up depends on the number of inflections of the curve and not on the number of averages taken. Thus, the exact number of plots making up an average becomes less important. These averages are used as a basis for constructing a smooth curve (Fig. 1).

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[3] Instead of yield any other data that can be expressed numerically such as estimates of insect population or heights or stand counts of plants may be corrected in this manner.

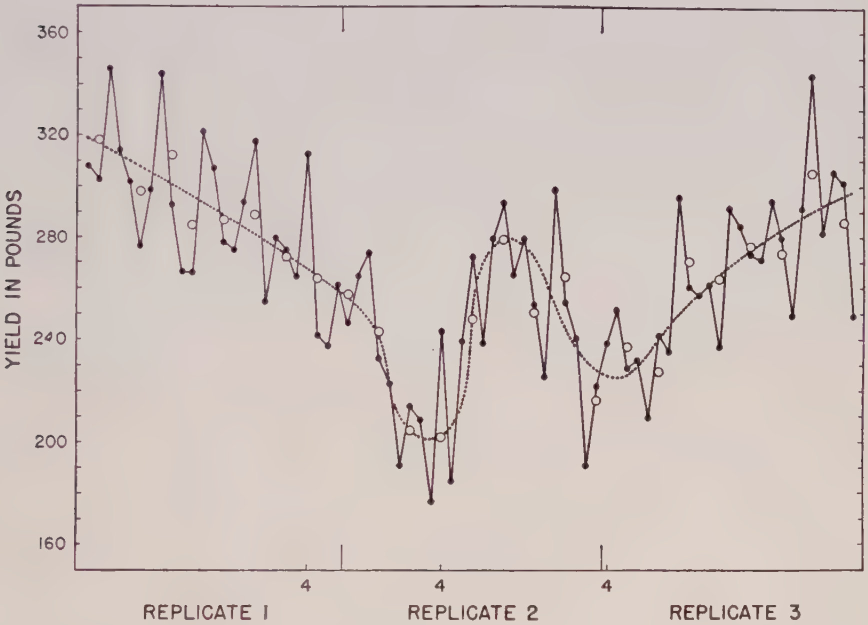

FIGURE 1. Yields, in pounds, independent of treatment for potatoes. Averages are open circles connected by smooth curve. Correction is made from smooth curve. Individual values are shown only for comparison and would not ordinarily appear.

The yield as given by the smooth curve at the location of the plot under consideration is designated as C.

$$\frac{\overline{Y}}{C} \times R = Y_g. \tag{ii}$$

In this formula, Y_g is the geographically corrected yield. Where it is possible to collect the data by portions of plots running at right angles to the long axis of the field[4] certain difficulties to be discussed later can be avoided. In this case, these portions are handled individually as R values. $\overline{Y}$ becomes the average for the field of portions this size and y becomes the average for each treatment of portions this size. When this is done G values are averaged for portions of adjacent plots for the values from which the curve is plotted. Thus if each plot consists of four rows of potatoes (*Solanum tuberosum* L.) each harvested separately, the calculations are conducted on each row individually and G values for the last two rows of one plot are averaged with G values of the first two rows of the next plot to give the points for which the curve is drawn.

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[4] An example is where each plot consists of four rows which run lengthwise of the plot and each row yield is determined individually.

Example

Assume the following:

Treatment A yields 25 lbs. per 100-ft. row under the same conditions where B yields 50 lbs., C–75 lbs., and D–25 lbs.

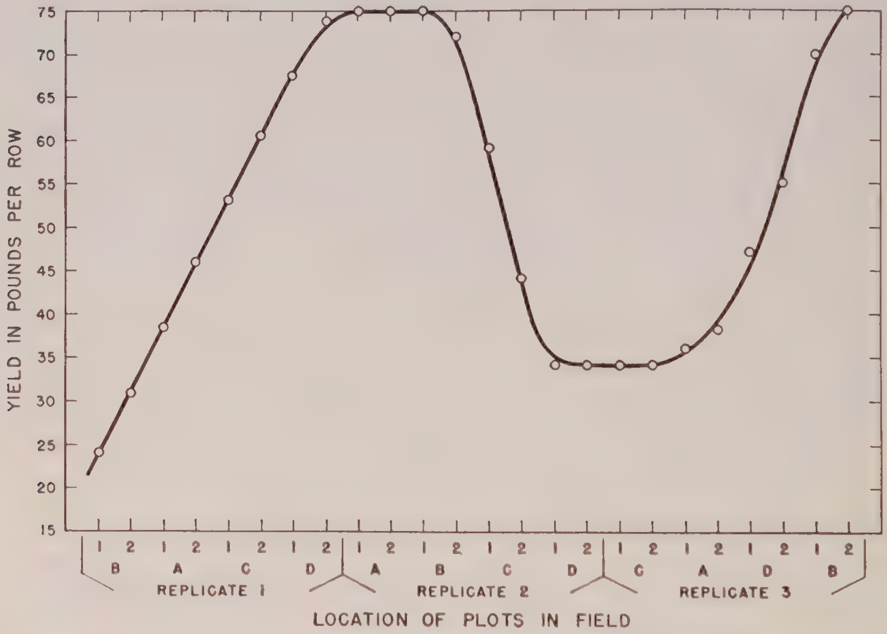

FIGURE 2. Hypothetical distribution of yield for example given in text. Treatments indicated by letter, each consists of rows 1 and 2.

A field is selected for the experiment where geographic variations in yield in a uniformity trial (if B had been used in the entire field) would give the yield contour shown in Figure 2. The design consists of two 100-ft. rows to make up a single plot and three replications. Randomization has arranged the plots in the order shown on the graph.

Now, the yields that would have been obtained in this experiment may be gotten by multiplying the value given in the graph by the appropriate fraction that the treatment yields compared with B. Thus, for Replicate 1 the values of rows 1 and 2, Treatment B, can be read directly from the graph, the values for rows 1 and 2, Treatment A, will be the graph values 38 and 46 multiplied by one-half, or 19 and 23 respectively, since by definition A yields 25 lbs. where B yields 50 lbs. Yields are read from open circles (Fig. 2) which are whole number values and consequently are not always centered on the hypothetical yield contour curve. These values are compiled in Table I.

TABLE I

YIELDS OBTAINED FROM FIGURE 2 AND KNOWN YIELDS OF A, B, C, AND D

	A		B		C		D	
	Row 1	Row 2	Row 1	Row 2	Row 1	Row 2	Row 1	Row 2
Repl. 1	19	23	24	31	80	91	34	37
Repl. 2	37	37	75	72	89	66	17	17
Repl. 3	18	19	70	75	51	51	24	27
Totals	153		347		428		156	
Grand Total							1,084	

It will be understood that so far this example has been concerned only with setting up a hypothetical experiment to obtain the yields given in Table I. Extreme geographic variation has been postulated (Fig. 2 shows three-fold variation between high and low yielding areas for the same treatment). No random variation has been introduced and for purposes of this example this is not necessary. From now on the values given in Table I will be treated exactly as if they had been yield data obtained in a field experiment.

Adding the values given for rows in Table I, these data become the values shown in Table II.

Thus, because of the effect of geographic variation on yield, none of the treatments in this experiment could have been differentiated by the usual statistical procedures.

Using formula (i) above and using row yields for R values, the G values become those given in Table III.

The field average per row $\overline{Y}$ is the sum of all the entries in Table I divided by the number of entries: $\frac{1084}{24}=45$. Decimals are dropped throughout. A false sense of precision might be obtained if they were carried. The y values for treatments A, B, C, and D (on a row basis) are respectively: 25, 58, 71, and 26. These are obtained by taking the treatment totals and

TABLE II

YIELDS BY PLOTS OBTAINED FROM TABLE I

	A	B	C	D	Total
Repl. 1	42	55	171	71	339
Repl. 2	74	147	155	34	410
Repl. 3	37	145	102	51	335
Totals	153	347	428	156	
Av. per plot	51	116	143	52	
Av. per row	25	58	71	26	

Analysis of variance

	Degrees of freedom	Sums of squares	Variance	Significance
Treatments	3	19,192	6,397	No
Replicates	2	891	445	No
Treatments × Replicates	6	8,732	1,455	
Total	11	28,815		

LSD for 19:1 odds for treatments (plot averages) 76.*

* Not to be used since analysis of variance indicates no significant differences between treatments in general.

dividing by the number of rows in each treatment $\left(\text{for A}: \frac{153}{6} = 25\right)$. Thus for row 1, Treatment A, Replicate 1, the numerical values in formula (i) become

$$\frac{45}{25} \times 19 = 34.$$

The data in Table III are used to construct Figure 3. The geographical location of the plots in the field is plotted on the abscissa as for Figure 2. The yield is plotted on the ordinate. Row 2 of each plot is averaged with row 1 of the adjacent plot to obtain the closed circles which are the basis for the construction of the smooth curve. Note that row 2 for any plot has

TABLE III

G VALUES OBTAINED FROM THE DATA OF TABLE I BY THE RELATION $\frac{\bar{Y}}{y} \times R = G$

	A		B		C		D	
	Row 1	Row 2	Row 1	Row 2	Row 1	Row 2	Row 1	Row 2
Repl. 1	34	41	19	24	51	58	59	64
Repl. 2	67	67	58	56	56	42	29	29
Repl. 3	32	34	54	58	32	32	41	46

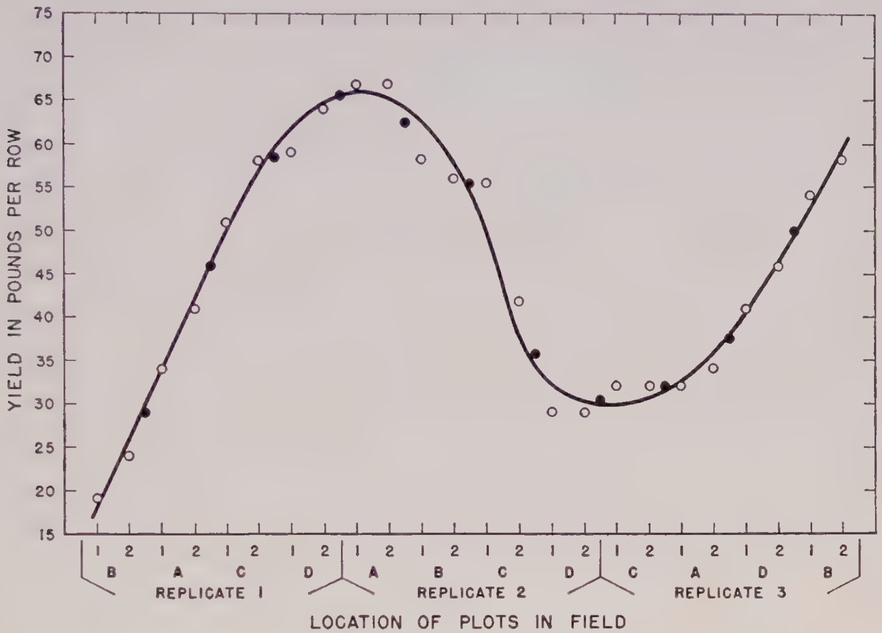

FIGURE 3. Curve for obtaining correction values for example. Open circles are calculated values from Table III. Closed circles are averages of two adjacent calculated values.

no other relation to row 1 of the succeeding plot except geographical juxtaposition. Since this is true and since the row values do not enter into the analysis of variance presented here, none of the degrees of freedom used in the analysis has been used here. This is a salient point of this method. Note also that the contour of this curve is essentially the same as the one presented in Figure 2. This is confirmation that the geographic variation can be taken out even when different treatments have been applied. Such confirmation is not readily obtainable in a field experiment since there the geographic variation, independent of treatment, is not known. The *C* values are now read from the graph and appear in Table IV.

TABLE IV

C VALUES READ FROM FIGURE 3

	A		B		C		D	
	Row 1	Row 2	Row 1	Row 2	Row 1	Row 2	Row 2	Row 2
Repl. 1	33	41	19	26	50	58	63	65
Repl. 2	66	65	62	58	50	40	32	30
Repl. 3	33	36	54	60	30	31	40	47

Using equation (ii) $\frac{\overline{Y}}{C} \times R = Y_a$ adjusted yield, the geographically corrected yields become those shown in Table V. For example for row 1, Treatment A, Replicate 1, the adjusted yield is $\frac{45}{33} \times 19 = 26$.

TABLE V

Geographically Adjusted Yields (Y_g) by Row

	A		B		C		D	
	Row 1	Row 2	Row 1	Row 2	Row 1	Row 2	Row 1	Row 2
Repl. 1	26	25	57	54	72	71	24	26
Repl. 2	25	26	53	56	80	74	24	25
Repl. 3	25	24	58	56	76	74	27	26
Totals	151		334		447		152	

Rows 1 and 2 are added together and the results are presented in Table VI. All treatments now differ significantly except A and D which were by original definition the same. The LSD 19:1 for the averages presented in Table II is 76. Since this example did not include random variation, the improvement in differences necessary for significance reflects only the success in removing geographic variation. An exact method would have removed all geographic variation in this example. The measure of the success of this approximation may be seen by comparing the treatment × replicate variation in the analysis of variance for Table II and that for Table VI. Note that the replicate variance has remained non-significant after the correction. In cases where replicate variation is significant before the correction, this method will reduce it to non-significance because replicate variation is in itself due to geographic variation as herein described.

DISCUSSION

This method is not a substitute for well-designed and well-planned experiments. In a small experiment, for example, one containing eight or less treatments, it is necessary that at least reasonably good average estimates for each treatment be made and this can only be assured by adequate replication. In larger experiments averages for one or more treatments may be in error and the correction still be useful; even here it is necessary that the average of the majority of treatments be good. The only variation that this method will reduce is a geographic variation and then only such variation that runs through several plots and can be fitted by some simple smooth curve. It is interesting that in use on potato plots the method has significantly reduced error variance for several years in Pennsylvania and West Virginia but only minor improvement was noted for an experiment on muck soil in Florida. Since conditions of the latter experiment were as nearly uniform as is ever found in field work this is to be expected.

A correction was run on a uniformity trial from published data [values read from Fig. 2 of West Virginia Bull. 189 (4)]. Treatments were dummied in with replication. After the correction, the data remained a uniformity trial but the variance of both dummy treatments and treatments×replicates had been reduced. This was done several times with the randomization of the treatments differing in each trial. The degree of reduction of variance varied between one-fourth and one-third of its former value in the several trials.

TABLE VI

GEOGRAPHICALLY ADJUSTED YIELDS BY PLOT

	A	B	C	D	Total
Repl. 1	51	111	143	50	355
Repl. 2	51	109	154	49	363
Repl. 3	49	114	150	53	366
Totals	151	334	447	152	1,084
Av. per plot	50	111	150	51	
Av. per row	25	55	75	25	

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Significance
Treatments	3	21,169	7,056	High
Replicates	2	17	8.5	No
Treatments×Replicates	6	69	11.5	
Total	11	21,255		

LSD 19:1 for treatments (plot averages) 6.7.

As an example, in one trial the uncorrected data gave a variance of 302 for the dummy treatments with 14 degrees of freedom and an error variance of 628 with 28 degrees of freedom. When these data were geographically adjusted the dummy treatment variance was 196 and the error variance 149. If 2 degrees of freedom were deducted for the initial inflection of the curve used for the correction and an additional degree of freedom for each additional inflection the error variance became 219 with 19 degrees of freedom.

In another test of the validity of the correction, treatment effect was removed as described above and the data analyzed before reinsertion of the treatment effect on the corrected yields. The results were those of a uniformity trial.

In some experiments conducted at State College, Pa., the curve constructed for the geographic correction has been a reasonably accurate profile of the field contour. Gullies running through the field were reflected by minima in the curve and a high spot by a maximum. Under different weather conditions such direct correlation might not be apparent.

The visual fitting of the curve is an obvious weakness of this method. In general, minor variations in position of the curve will make little difference in results. However, in areas where the curve has a very steep inflection minor positional differences can have great effect. In comparing Figures 2 and 3, and considering only relative shape of curves, neglecting numerical values which are not directly comparable, it will be seen that the curve in Figure 3 does not reach the maximum or minimum values of that of Figure 2. This feature, together with rounding the approaches to these extremes, is a result of the method of averaging used to characterize the curve.

If averages of plots are used to determine the points on the curve as is done in the case shown in Figure 1, there is a question as to the number of degrees of freedom used in drawing the curve. The authors cannot answer this question precisely but believe that a conservative approach is to deduct from the degrees of freedom available for error one more than the number of inflections of the curve. As the experiment increases in treatments or replicates, such a reduction will not materially enlarge error variance. As has been stated above, the method used in the example avoids this difficulty.

A field experiment conducted on the effect of spray treatments on potatoes was so designed that it could be treated either as simple replicates or as a triple lattice. This experiment consisted of 21 treatments replicated three times. Each plot consisted of four rows of potatoes, each row 120 ft. long. The treatments were arranged linearly in a single strip on a gentle slope. The rows (and treatments) ran across the strip, *i.e.*, at right angles to the long dimension of the field. Analyses of variance were conducted on these data by the following methods: (A) using replicates × treatments as error; (B) using the triple lattice design and the appropriate error term (1); (C) using the geographic correction on plot averages (groups of four) then replicates × treatments as error for the corrected data; (D) as for (C) except rows were used for the geographic correction, the last two rows of each plot were averaged with the first two rows of the succeeding plot. The results are presented in Table VII.

The advantage of this method is well demonstrated by the decreased difference necessary for significance as shown in Table VII. Another value of the correction for large experiments can be seen in Figure 1 for Treatment 4 that occurred by chance in low yielding areas in all three replicates and would in the usual treatments be rated lower than its actual value. This was taken care of by the correction.

Experiments were conducted in which no correlation could be determined between insect or disease control, or both, and uncorrected yield

TABLE VII

ERROR TERMS AND LEAST SIGNIFICANT DIFFERENCE FROM ANALYSES OF VARIANCE CONDUCTED ON RESULTS OF FIELD EXPERIMENT ON FUNGICIDAL SPRAYS CONDUCTED AT STATE COLLEGE, PA., ON KATAHDIN POTATOES—1946

Bases of analyses as explained in text	Error variance	Least significance difference in lbs. per plot*
A	2,840	87
B	2,129	75
C	672	42
D	206	24

* 19:1 odds.

of potatoes. After the correction was made, both insect and disease control separately gave significant correlations with yield. This correlation was significantly improved when multiple regressions were run with insect and disease controls as the independent variables and corrected yield as the dependent variable.

SUMMARY

A method is described which minimizes the geographic variation found in so many field experiments and an example is given. Geographic variation is defined as any organized variation which affects the data such as soil type, soil profile, moisture, or weed population variation. It is shown that this method makes possible more precise comparisons than are possible on uncorrected data. The method does not impose limitations on number of treatments or replicates and has been found to minimize geographic variation better than existing methods.

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A LEAF IMMERSION TECHNIQUE FOR STUDYING THE ABSORPTION AND TRANSLOCATION OF CHEMICALS IN PLANTS[1]

Lawrence J. King[2]

INTRODUCTION

In a program where large numbers of chemicals are screened for general growth response studies, or for herbicidal action, many rapid testing techniques are desirable. Any one test has a decided advantage if it provides information on a number of different plant growth responses, and at the same time helps to elucidate the problems of absorption and translocation. The leaf immersion technique fulfills many of these requirements. This technique is a method whereby terminal portions of intact, uninjured leaves, are immersed in liquid preparations of chemicals, with responses measured in terms of the distance of visible injury in the immersed leaf, and in growth rate measurements of the treated plant. In such rapidly developing fields as plant chemotherapy, plant hormones, and herbicides, information on the absorption and translocation of chemicals under test is essential. Similar information is of value in the development of effective fungicides and insecticides.

The spray method of applying chemicals to the surfaces of plants has long been in use as a standard technique. This method has one disadvantage in that a complete coverage of the plant with resulting rapid toxic response frequently obscures the actual or potential responses of the plant. Localized applications of chemicals have certain definite advantages, and many different methods have been described. Lanolin or "Carbowax" applications as smears or as pellets have been used (2). The pot soil treatment method as used by Hitchcock and Zimmerman (6) provides information on the uptake of chemicals from the soil. Their method of chemical treatment of excised tomato plants is a valuable technique as well (12). Miller (7) has developed a split stem technique where in tomato plants an auxiliary root system was developed, thus providing a more direct method of introducing chemicals into plants without injury to the established root system. Plant injection methods have been extensively used by Roach (8).

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The kidney bean single-droplet water test and single-droplet oil test as described by Thompson *et al.* (11) are useful for testing physiologically active substances. A thread method for introducing small quantities of chemicals into plants has been described by Felber (5) and by Roberts (10). Anderssen (1) in studying the chlorosis of plum trees (*Prunus salicina* Lindl. var. Kelsey) immersed intact, uninjured leaves in copper sulfate solutions (0.3 p.p.m. Cu), and noted the disappearance of the chlorosis. The technique as described here involves for the most part the study of chemicals at toxic concentrations, and places the injury or growth responses of the treated plants on a quantitative basis.

MATERIALS AND METHODS

Corn (*Zea mays* L. var. Early Golden Orange Dent) seedlings grown in 3 inch pots, and from 15 to 25 days old, were used in the majority of the tests. A recently emerged leaf was selected—not any of the early leaves for they soon wither and die on the plant. It is true that these young leaves are quite immature and more easily penetrated by chemicals than are older leaves. However, chemicals proving interesting on the young plants can be later tested on older corn leaves.

The leaves were bent over and the terminal 9 cm. portion was immersed in 100 ml. of solution contained in a 150 ml. beaker. The leaves were held in position against the wall of the beaker by a small piece of cardboard folded on the edge of the beaker and held by a paper clip.

Corn leaves of young plants thus utilized provide an excellent way of studying the absorption and translocation of many types of chemicals, mixtures of chemicals, and formulations. Both water-soluble and water-insoluble materials can be studied by this technique. The latter may be prepared as emulsions and handled in the same manner as the water-soluble preparations.

The long linear leaf is well suited to measuring the visible injury responses of the chemical under study, and for measurement at definite time intervals. Since accurate measurements along the curved leaf even with a flexible rule are difficult to make, all measurements were made to the nearest cm. Measurements were made from the line of immersion of the leaf back to the base of the blade (excluding the sheath). For chemicals not visibly injurious to the immersed leaf, another measuring method may be used. This method involves, in corn, the selection of a leaf just appearing and measuring its growth rate throughout the test period. Should the chemical prove harmful to the growth of the plant, it could be detected by this type of measurement.

While for preliminary screening work a single plant may be used, if information on translocation in the rest of the plant is desired, it is better to use at least two plants and preferably four. The detailed study on sodium arsenite indicates the amount of variation that may occur.

Young potted tomato plants (*Lycopersicon esculentum* Mill. var. Bonny Best) were also used in some of the tests. A leaf about one-half the way up the axis, generally the fourth leaf, was selected for immersion. Only the terminal leaflet was immersed. The leaf portion studied for injury is much shorter than in corn, averaging about 16 cm., while in corn the length averages about 32 cm. Measurements on tomato leaves were made similarly to those on corn leaves. For those chemicals not visibly injurious to the leaves, measurements can be made on the growth rate of the plant axis.

The plants were all grown under average greenhouse conditions. Also all tests were conducted under the same conditions. All pots were watered uniformly. Crafts (4) has shown the relationships between water deficits in plants and the effectiveness of arsenical herbicides—the greater the deficit the greater the injury.

The leaf immersion tests described below include a preliminary testing of miscellaneous inorganic and organic compounds that have been used as herbicides, and a detailed study of leaf injury and growth rates on several of these. These tests illustrate the methods and show the possibilities to be achieved with the use of the leaf immersion technique.

TESTS ON MISCELLANEOUS CHEMICALS

In Table I are presented tests on single corn and tomato plants of some inorganic herbicides, some organic herbicides, and one oil. Corn plants were about 15 days old, and 9 cm. of the terminal portion of a leaf was immersed in the solutions. The terminal leaflet of a leaf (frequently the fourth leaf) about one-half the way up the axis of young potted tomato plants was immersed in the solutions also. In both plants measurements on the maximum distance of visible injury in the leaves were taken at 24 and 48 hours, and again at 7 days.

In the corn plants at the end of 48 hours copper sulfate, potassium chlorate, sodium arsenite, zinc chloride, and sulfuric acid had all been absorbed and translocated the entire length of the immersed leaf. By the seventh day all of these plants were dead with the exception of the sulfuric acid and zinc chloride treated plants.

In the tomato series at 48 hours only three compounds, sodium arsenite, sulfuric acid, and "Sinox" (sodium dinitro-*o*-cresylate), had injured the entire length of the immersed leaves. By seven days potassium chlorate, potassium dichromate, potassium chromate, ammonium thiocyanate, sodium arsenite, sulfuric acid, and "Sinox" had injured the entire length of the immersed leaf. "Dowspray" (dinitro-*o*-*sec*-butylphenol in oil) passed through and killed the top of the plant. This material is an oil formulation. Kerosene was tested, but it injured only a little more than one-half the distance of the corn leaf, while in tomato it injured nearly the entire length of the immersed leaf.

TABLE I
CORN AND TOMATO LEAF IMMERSION TESTS ON INORGANIC AND ORGANIC COMPOUNDS AT CONCENTRATIONS OF 1.0 PER CENT
(VALUES ARE MEASUREMENTS OF SINGLE PLANTS)

Compound	Distance of injury in cm.					
	Corn			Tomato		
	24 hrs.	48 hrs.	7 days	24 hrs.	48 hrs.	7 days
Ammonium thiocyanate	18	20	31 EL	6	8	14 EL
Boric acid	0	8	33 EL	0	10	13
Copper sulfate	23 EL	23 EL	PD	18	18 EL	18 EL
Potassium chlorate	0	31 EL	PD	9	18	18 EL
Potassium chromate	20	22	30 EL	4	7	17 EL
Potassium dichromate	10	15	26	5	9	14 EL
Sodium arsenite	0	28	PD	18	18 EL	18 EL
Sodium chloride	0	19	34 EL	0	0	6
Sodium thiocyanate	0	20	31 EL	0	7	9
Sulfuric acid	33	34 EL	34 EL	18	20 EL	20 EL
Zinc chloride	23	28 EL	EP	3	6	7
"Dowspray 66 Improved"*	8	8	EL	7	7	PD
Sodium dinitro-*o*-cresylate ("Sinox")	6	23	23 EL	12	15 EL	15 EL
Kerosene (100%)	17	17	19	12	12	12
Water control	0	0	0	0	0	0

EL = entire leaf; EP = entire plant; PD = plant dead.
* Contains dinitro-*o*-*sec*-butylphenol.

LEAF INJURY AND GROWTH MEASUREMENTS ON CORN LEAVES FOR A SERIES OF COMPOUNDS

In Table II are the results of some tests on ammonium sulfamate ("Ammate"), sodium dinitro-*o*-cresylate ("Sinox"), and sulfuric acid. The tests were on corn plants in replicates of four, and injury measurements and growth measurements on a selected leaf were taken at 24 hours and at 7 days.

In the replicated tests in the majority of the leaves neither "Ammate" nor "Sinox" was translocated the entire length of the immersed leaves, and consequently not into the rest of the plant. As indicated later, sodium arsenite moves rapidly through corn leaves and into the rest of the plant. Sulfuric acid in corn rarely appears to be translocated beyond the immersed leaf. The growth rates of the marked leaves were not greatly reduced by these concentrations of 1 per cent and below, at least this was not nearly the reduction that occurred in the "Ammate" series at 5 and 10 per cent where growth was 35 and 40 per cent of that of the controls.

LEAF INJURY AND GROWTH MEASUREMENTS OF A SODIUM ARSENITE SERIES

Sodium arsenite has long been known as a compound highly toxic to plant tissues (*3*). Extensive studies have been made by Robbins, Crafts,

TABLE II
CORN LEAF IMMERSION TESTS ON A SERIES OF COMPOUNDS
(VALUES ARE AVERAGES OF FOUR REPLICATES)

Compound and concn., %		Average distance of injury in cm.		Average growth in length of selected leaves			
		24 hrs.	7 days	24 hrs.		7 days	
				Av. cm.	% of control	Av. cm.	% of control
Ammonium sulfamate	10	20.8	31.5	9.3*	46	11.3	35
Ammonium sulfamate	5	20.3	32.3	10.8*	54	12.8	40
Ammonium sulfamate	1	2.0	37.3	8.0	—	27.5	87
Water control		0	0	20.0*	100	32.3	100
Water control		0	0	8.0	100	31.6	100
Sodium dinitro-*o*-cresylate ("Sinox")	1	1.8	22.8	8.1	94	29.3	92
Sodium dinitro-*o*-cresylate ("Sinox")	0.5	7.0	34.8	7.8	91	25.1	79
Sodium dinitro-*o*-cresylate ("Sinox")	0.1	1.8	18.8	8.0	93	28.6	90
Water control		0	0	8.6	100	31.8	100
Sulfuric acid	2	16.8	27.8	10.0	93	23.0	71
Sulfuric acid	1	18.3	29.5	9.3	86	26.0	80
Sulfuric acid	0.5	12.8	23.0	9.5	88	28.0	87
Water control		0	0	10.8	100	32.3	100

* Values here are for measurements at 5 days rather than 24 hours.

and Raynor (9) on the arsenicals as herbicides, and soil sterilants. Leaf immersion studies with this compound illustrate well the possibilities of this type of test.

A sodium arsenite series at four concentrations, 10, 5, 2, and 1 per cent, was tested with four plants replicated for each concentration. Leaves were immersed as previously described. On each plant an emerging leaf was marked for growth measurements. Leaf injury measurements were made at 24 and 48 hours, and at 6 days, and growth measurements on the marked leaf as well. The average values of these two measurements for the four plants at the 24-hour and the 6-day period are given in Table III.

In the first 24 hours in the 10 per cent series, the sodium arsenite was absorbed and translocated through the entire length of the immersed leaves in the four replicates, and in three of the plants it had moved to all parts of the plants. At the concentrations of 1, 2, and 5 per cent in practically none of the leaves had the chemical injured the entire length. At 48 hours, however, the chemical had injured the entire length of all of the immersed leaves. At 6 days, at 10 per cent, two plants were dead and two others were nearly so. At concentrations of 1 and 2 per cent the chemical

TABLE III

LEAF INJURY AND GROWTH MEASUREMENTS IN CORN PLANT LEAVES IMMERSED IN SOLUTIONS OF SODIUM ARSENITE (VALUES ARE AVERAGES OF FOUR REPLICATES)

Concn. of sodium arsenite %	Average distance of injury in cm.		Average growth in length of selected leaves			
			24 hours		6 days	
	24 hrs.	6 days	cm.	% of control	cm.	% of control
10	25.5 EL	25.5 EP	1.4	22	3.8	18
5	13.3	28.3 EL	3.8	60	4.5	22
2	15.5	23.8 EL	4.8	76	9.3	45
1	14.8	24.5 EL	6.0	96	15.0	73
Water control*	0	0	6.3	100	20.5	100

EL = entire leaf injured in all plants; EP = entire plant injured in all replicates.
* Average length of leaves less immersed tips was 28.5 cm.

had apparently moved throughout the entire plant in only two individuals. The growth rate measurements taken on young elongating leaves in the 10 per cent series at 24 hours show only 22 per cent of that of the control leaves (see Fig. 1).

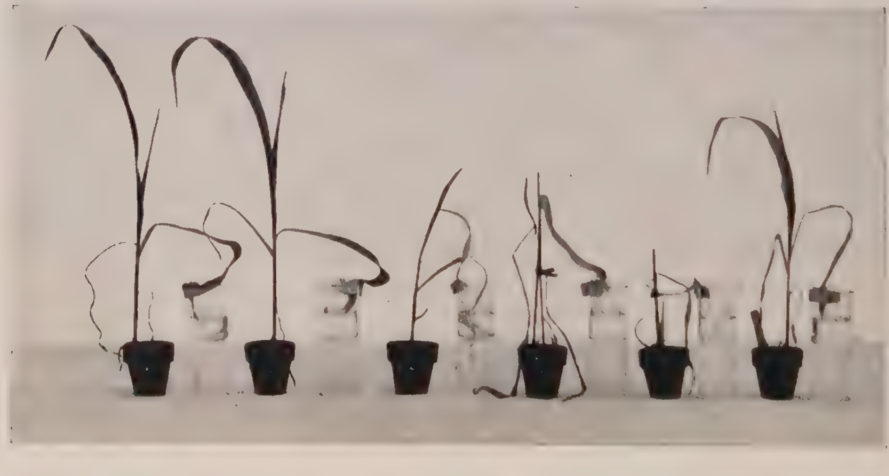

FIGURE 1. Leaf immersion test with sodium arsenite using corn plants. Left to right: water control, water control, and four replicates in which the leaves were immersed in 10 per cent solutions of sodium arsenite. Picture taken at seven days.

SUMMARY

The leaf immersion technique as described is a method whereby terminal portions of intact, uninjured leaves, are immersed in liquid preparations of chemicals, with responses measured in terms of the visible injury distance in the immersed leaf, and in growth rate measurements of the treated plant.

Immersion tests were conducted with potted corn and tomato plants. Selected leaves were immersed in solutions of a series of twelve inorganic compounds that have been used as herbicides, and two standard organic herbicides.

From the evidence of visible injury, absorption and translocation through the entire distance of the immersed corn leaf occurred in the majority of the chemicals tested at a concentration of 1 per cent after a period of 7 days. Translocation into the rest of the plant occurred less readily, and was observed in the tests with copper sulfate, potassium chlorate, and sodium arsenite. The results with immersed tomato leaflets were similar to those of corn, except that translocation throughout the entire plant rarely occurred.

A detailed study was made of the absorption and translocation of sodium arsenite in immersed corn leaves. In the first 24 hours at a concentration of 10 per cent, sodium arsenite was absorbed and translocated through the entire distance of the immersed leaves of the four replicates, and in three of the plants it had moved to all parts of the plants. At the concentrations of 1, 2, and 5 per cent in practically none of the immersed leaves had the chemical injured the entire distance. At 48 hours, however, the chemical had injured the entire length of all of the immersed leaves.

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ACTIVATION OF 2,4-D BY VARIOUS ADJUVANTS[1]

A. E. Hitchcock and P. W. Zimmerman

For many years it has been a common practice to add various adjuvants to insecticidal and fungicidal spray solutions for the purpose of increasing their effectiveness. Herbidices are not an exception to this rule, and consequently weed-killing solutions used in practice, and also experimentally, generally contain carrier ingredients which are designed to increase the effectiveness of the spray solutions over that of a water solution of the principal active ingredient. The greater effectiveness of a hormone solution containing a Carbowax carrier, as compared with a water solution of the same hormone, was reported previously (12, p. 332). Other carriers for plant hormones have also been used in this laboratory and elsewhere, such adjuvants falling under the general classification of spreaders, solubilizers, stickers, emulsifiers, and penetrants. However, the use of more than one active ingredient in herbicides has received much less attention than the carriers which have been used with a single active ingredient.

In some of the earlier work with hormones it was found that mixtures containing two or three root-inducing hormones were more effective than any one used separately at a concentration equivalent to the total concentration of the mixture (4, 5, 6). Certain non-hormone compounds (vitamin B_1 and B_6) increased the root-inducing activity of indolebutyric acid (4). Similar synergistic effects were obtained with mixtures of hormones used to increase fruit set and induce the development of parthenocarpic fruit (12, p. 332). In other tests (unpublished) two-substance mixtures of the three possible combinations of 2,4-D (2,4-dichlorophenoxyacetic acid), 2,-4,5-trichhlorophenoxyacetic, and 2-methyl-4-chlorophenoxyacetic acids were more effective than any of the individual components for killing tomato and several kinds of weeds in greenhouse experiments. The weeds were species of the following genera: *Hieracium*, *Prunella*, *Plantago*, *Taraxacum*, *Stellaria*, and *Hydrocotyle*. A mixture of 2,4,5-trichlorophenoxy and 2-methyl-4-chlorophenoxyacetic acids was the most effective in these tests. In field tests a mixture of 0.1 per cent each of 2,4,D and α-naphthaleneacetic acid was more effective than 2,4-D in preventing regrowth from treated dandelion plants (7, p. 491).

Recent greenhouse tests have shown that lethal and non-lethal concentrations of contact herbicides used in combination with 2,4-D caused quicker killing of tomato test plants than either of the two components used separately at the same concentration as in the mixture. Differences in

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time of killing tomato plants were equivalent to or greater than those obtained with spreader solubilizers such as Tween #20 (hereinafter referred to as Tween) and polyethylene glycol 400 monooleate. These additive effects were obtained also with similar solutions containing 1 per cent Tween in addition to the contact herbicide and 2, 4-D. Other workers have also reported activation of 2,4-D by substances which have been used as contact herbicides (1, 8, 9, 10).

The present report describes the results obtained with Benoclor 3C (manufactured by the Cloroben Corp., Jersey City, N. J.) and certain other preparations or compounds used in combination with 2,4-D. Benoclor 3C (hereinafter referred to as Benoclor) is an emulsified form of Benoclor 3 which contains chlorinated benzenes as the active ingredients. The high specific gravity of Benoclors has made their use valuable for eradicating submerged aquatic plants particularly in irrigation ditches and canals (2, 3). Considering this characteristic together with claims for the relatively short-time residual effects (a few weeks) of Benoclor indicated that its use in combination with 2,4-D might cause a more complete killing of submerged parts of such aquatic plants as water hyacinth and alligator weed than 2,4-D used alone. Tests with tomato plants and a limited number of tests with volunteer seedlings of crab grass, purslane, and smartweed indicated that mixtures of Benoclor and 2,4-D may prove more effective than 2,4-D on land plants.

Some of the non-hormone preparations or compounds which functioned as activators of 2,4-D for killing plants also served as activators of 2,4-D for inducing hormone types of curvature responses. Conversely, relatively low concentrations of 2,4-D (0.00001 to 0.0001 per cent) activated a non-hormone compound (ammonium thiocyanate) in causing damage to tomato foliage and inhibition of growth even though these low concentrations of 2,4-D did not inhibit growth or kill leaf tissue when used alone.

MATERIALS AND METHODS

In view of the importance of available materials for use in practice, 2,4-D was used in combination with proprietary preparations as well as with known compounds. Considering the wide range in the class of compounds which increased the herbicidal activity of 2,4-D, it is believed that a knowledge of the principal active ingredients of proprietary preparations is sufficient for interpreting the results of the present experiments. According to directions for an antidote on the label, Hammond's Weed Killer contains an arsenical as the active ingredient. The active constituents in Benoclor are chlorinated benzenes (2, 3). DuPont's Ammate contains ammonium sulfamate as the active ingredient. The aromatic hydrocarbon oil is of the type used as a carrier for esters of 2,4-D, but the specific characteristics of this oil are not known.

The 2,4-D used in combination with Benoclor was 2,4-Dow Weed Killer containing 20 per cent 2,4-D acid equivalent in the form of an amine salt. Results of these tests are recorded in Tables I, II, and III. 2,4-Dow Weed Killer was also used in combination with diallyl maleate for which the results appear in Table V. Results in Table IV are based on tests in which the 2,4-D was a purified acid (m.p. 140° C. and 32.3 per cent chlorine) made up for use in the form of a sodium salt by adding the equivalent quantity of NaOH.

Bonny Best tomato (*Lycopersicon esculentum* Mill.) four to five inches in height was used as the principal test plant. Plants of this size generally died in 8 days when sprayed with 0.1 per cent 2,4-D. Except when otherwise noted, treatments were made in triplicate. A tomato plant was considered dead on the day when the stem and all leaves were flaccid or shriveled. No distinction was made between differences occurring on the same day, so that plants which died in the morning of a given day were rated the same as those which died in the late afternoon.

Volunteer seedlings of crab grass (*Digitaria* sp.), purslane (*Portulaca oleracea* L.), and annual smartweed (*Polygonum* sp.) which were germinated and grown in field soil were stored in flats in cold frames during the fall and early winter and moved to the greenhouse during February. At the time of spray treatment the stage of growth of weed seedlings was as follows: Crab grass in pre-heading stages with one to four leaves, purslane with one or two pairs of leaves, and smartweed mostly in the pre-flower bud stage and not branched.

RESULTS AND DISCUSSION

HERBICIDAL ACTIVITY

2,4-D and Benoclor. In preliminary experiments with Benoclor in combination with 0.1 per cent 2,4-D it was found that mixtures of these two preparations caused quicker killing of tomato test plants than either preparation used alone at the same concentration as in the mixture. Successive treatments whereby one spray was applied 24 hours after the other caused quicker killing, regardless of which preparation was applied first, than either spray treatment, and the killing was also quicker than that resulting from two successive applications of 2,4-D. The reduction in time to kill ranged from 1 to 7 days depending upon the concentration of Benoclor and the kind of combination treatment.

Treatment of tomatoes with 32 per cent Benoclor caused wilting of leaflets within 10 minutes, wilting of petioles in 24 hours or less, and collapse of the stem at soil level in 3 days. In contrast, 10 per cent Benoclor caused only a slight initial damage to foliage, but induced stem collapse in 1 day. Presumably the leaf tissue was killed so rapidly by 32 per cent Benoclor that transport of the stem-collapse factor or factors from leaves to stem was interfered with. However, the plants were killed 6 days sooner by the 32 per cent solution than by the 10 per cent solution. The lowest concentration of Benoclor (3.2 per cent) caused stem collapse in 2 days but failed to cause permanent wilting and did not kill the plants which recovered and resumed growth after the production of a new root system in much the same manner as a cutting. All three combination treatments with 3.2 per Benoclor and 0.1 per cent 2,4-D killed the test plants in 4 to 6 days as compared with 12 days for a single treatment with 2,4-D and 9 days for two successive treatments with 2,4-D.

Considering the highly toxic action of Benoclor at concentrations greater than 3.2 per cent, additive effects with 0.1 per cent 2,4-D were expected and they were obtained, even with 32 per cent Benoclor. These results show that a toxicant such as Benoclor does not prevent 2,4-D from causing a considerable additional killing effect. In later tests greater than additive effects were obtained also with non-lethal concentrations of Benoclor which did not cause stem collapse (0.32 and 1.0 per cent). Results of these tests are shown in Tables I and II and some of the responses on tomato are shown in Figures 1 and 2.

The average number of days required to kill three tomato plants is expressed as the nearest whole number in the body of Table I. For analysis of the data in the lower section of Table I the error term chosen consisted of the sum of the mean squares for replicates and for the second order interaction, exclusive of the 2,4-D controls. This combined error term (0.864 with 62 degrees of freedom) yielded a least significant difference (L.S.D) of 1.5 at the 5 per cent level. The error term chosen for mixtures alone, including the 2,4-D controls, was the sum of the mean squares for replicates (0.528) and for the first order interaction (3.36). The combined error term in this case (1.095 with 30 degrees of freedom) gave an L.S.D. of 1.74. A difference of 2 days between the average values in the lower section of Table I may therefore be regarded as significant at the 5 per cent level.

Results in the lower section of Table I and the responses illustrated in Figures 1 and 2 show that differences in the time of killing tomato plants varied with the concentration of 2,4-D and Benoclor, and also with the kind of combination treatments. The decrease in time to kill which resulted from an increase in concentration was about the same for 2,4-D and Benoclor in mixtures, as indicated by the column and line totals in Table I. This did not hold for successive treatments in which case Benoclor caused a greater difference than 2,4-D. It is to be noted, however, that the dosage ratio was greater for Benoclor than for 2,4-D. Some of the differences just described are illustrated in Figures 1 and 2. The lesser effectiveness of the successive treatments is due partly to a delay of 1 day in completing the treatment. If 1 day is subtracted from each of the average values for successive treatments, the results then would be more nearly comparable to those for mixtures (Table I).

Mixtures of 1.0 per cent Benoclor and from 0.05 to 0.2 per cent 2,4-D were significantly more effective for killing the test plants than the 2,4-D control solutions (Table I). These particular mixtures were from two to four times as effective as 2,4-D controls and three to ten times as effective as the Benoclor controls (footnote**, Table I). The corresponding successive treatments were less effective than the mixtures. If, as previously suggested,

TABLE I

ADDITIVE EFFECTS OBTAINED WITH 2,4-D IN COMBINATION WITH NON-LETHAL CONCENTRATIONS OF BENOCLOR 3C. RESPONSES ON TOMATO EXPRESSED AS THE AVERAGE NUMBER OF DAYS TO OCCUR

Per cent Benoclor 3C (B 3C)	Applied as mixtures					Successive treatments, one day between							
						2,4-D applied first				B 3C applied first			
	Per cent 2,4-D*				Line totals	Per cent 2,4-D*			Line totals	Per cent 2,4-D*			Line totals
	0.0	0.05	0.1	0.2		0.05	0.1	0.2		0.05	0.1	0.2	
All leaves wilted permanently													
0.0	—	10	7	6	23	—	—	—	—	—	—	—	—
0.32	**	8	7	5	20	8	8	7	23	9	8	7	24
1.0	**	5	5	4	14	6	5	5	16	7	6	5	18
3.2	**	5	4	4	13	6	5	5	16	5	4	5	14
Totals†		18	16	13	47	20	18	17	55	21	18	17	56
Stem collapse at soil level													
0.0	—	10	8	8	26	—	—	—	—	—	—	—	—
0.32	**	10	8	5	23	10	9	8	27	11	9	9	28
1.0	**	4	5	4	13	7	7	6	20	6	6	6	18
3.2	3	4	3	3	10	5	5	6	16	4	2	4	10
Totals†	3	18	16	12	46	22	21	20	63	21	17	19	56
All plants killed‡													
0.0	—	11	8	8	27	—	—	—	—	—	—	—	—
0.32	**	11	9	5	25	10	9	8	27	11	10	9	30
1.0	**	6	6	5	17	8	7	7	22	8	8	7	23
3.2	**	7	4	4	15	6	6	6	18	7	5	5	17
Totals†		24	19	14	57	24	22	21	67	26	23	21	70

* 2,4-Dow Weed Killer (amine salt).

** At these concentrations Benoclor failed to cause permanent wilting or death of plants, but a 10 per cent solution killed all plants in 4 days as in the case of 0.4 per cent 2,4-D.

† Exclusive of 2,4-D controls.

‡ L.S.D. for averages = 2 days, and for line and column totals = 3 days.

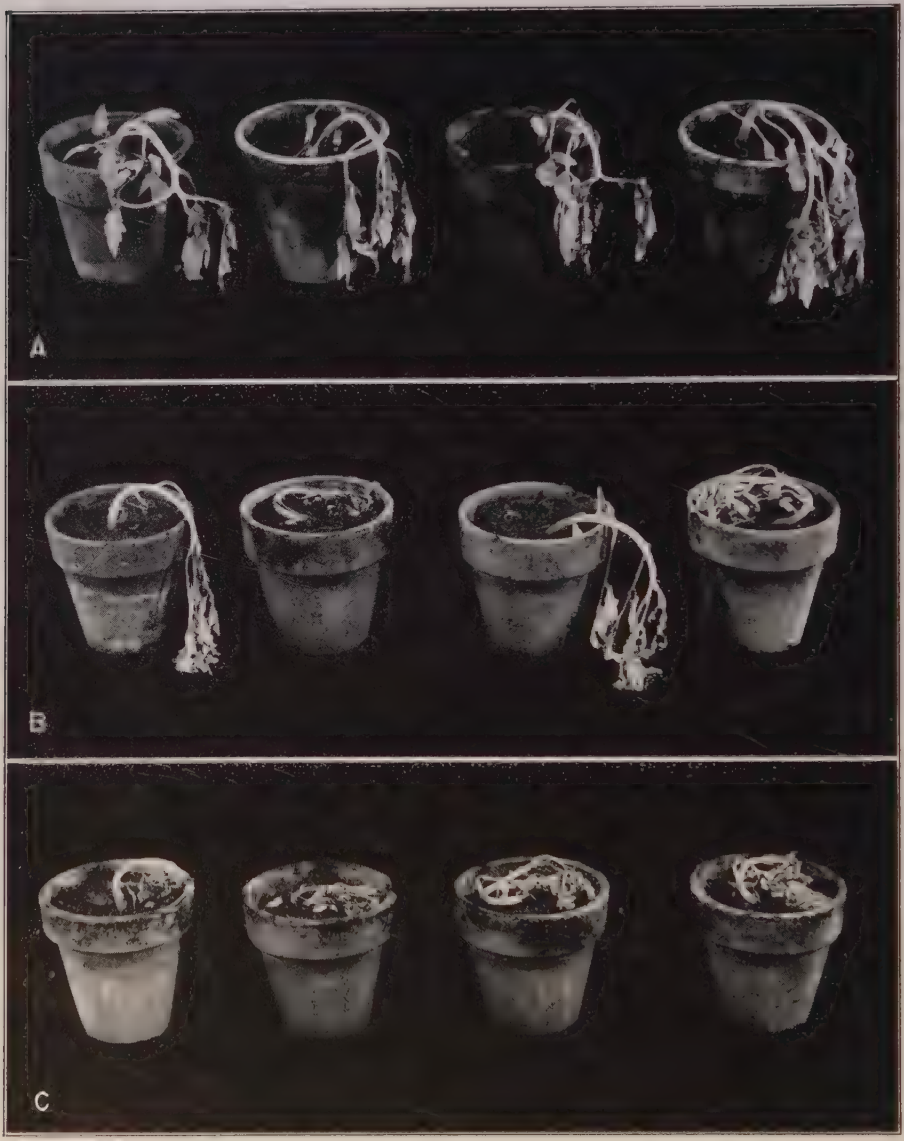

FIGURE 1. Bonny Best tomato one week after spraying with 2,4-D and with Benoclor in combination with 2,4-D. Plant on left sprayed with 2,4-D as follows: A, 0.05; B, 0.1; and C, 0.2 per cent solutions. Three plants on right treated as follows (L. to R.): Mixtures of 2,4-D and Benoclor, successive treatments with 2,4-D applied first and Benoclor after 24 hours, and a similar successive treatment with Benoclor applied first. For mixtures and successive treatments the concentration of 2,4-D was 0.05 per cent, and for Benoclor the concentration was as follows: A, 0.32, B, 1.0; and C, 3.2 per cent. Additive effects obtained with increasing non-lethal concentrations of Benoclor in combination with 0.05 per cent 2,4-D were similar to those obtained with increasing concentrations of 2,4-D used alone.

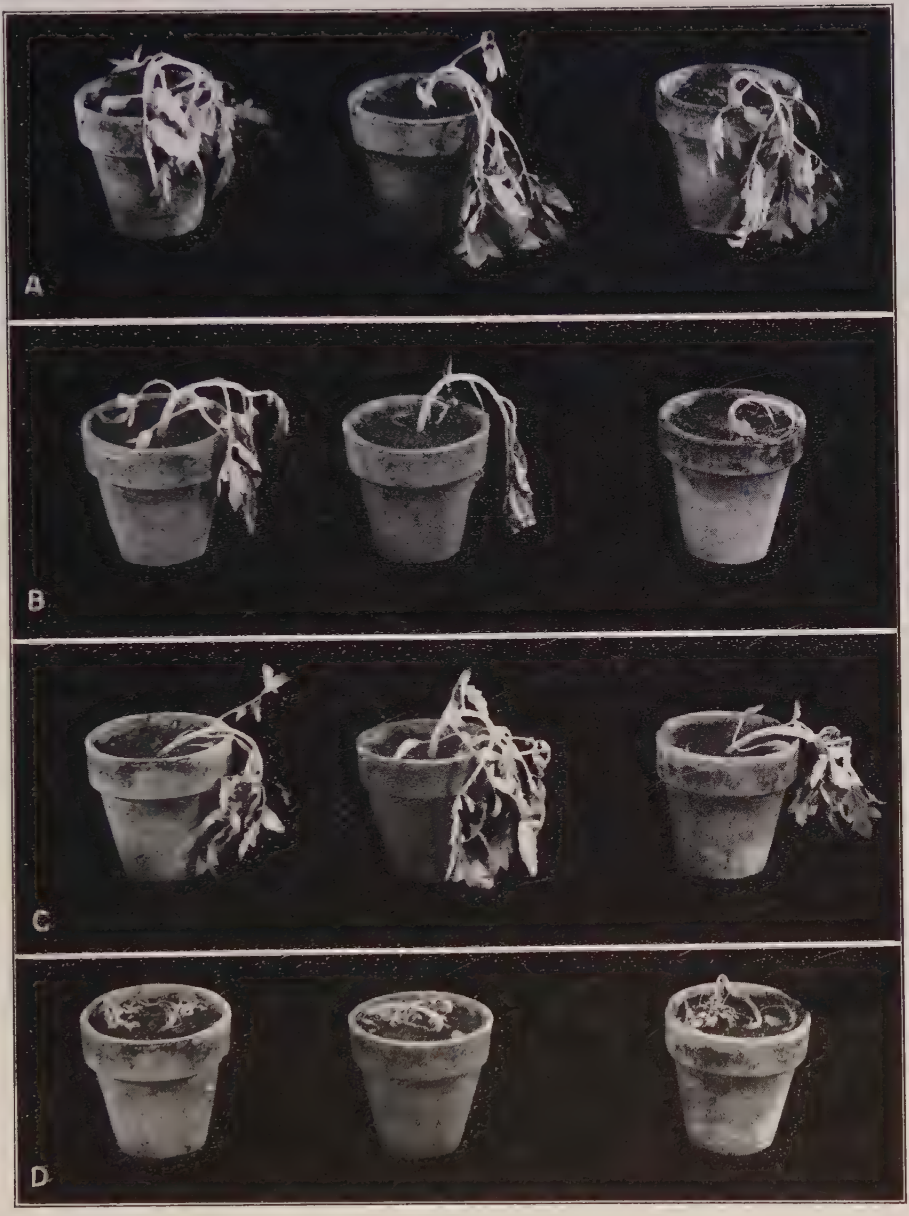

FIGURE 2. Appearance of Bonny Best tomato on the third (A and C) and seventh (B and D) days after spray treatment with 2,4-D and with Benoclor in combination with 2,4-D. Concentration of 2,4-D (L. to R. in rows A and B): 0.05, 0.1, and 0.2 per cent. Combination treatments (L. to R. in C and D): Mixtures of 0.1 per cent 2,4-D and 3.2 per cent Benoclor, successive treatment with 0.1 per cent 2,4-D applied first and 3.2 per cent Benoclor after 24 hours, and a similar successive treatment in the reverse order. Additive effects of 3.2 per cent Benoclor 3C in combination with 0.1 per cent 2,4-D were greater than those obtained with 0.2 per cent 2,4-D used alone.

1 day is subtracted from the average values for successive treatments (Table I), then five of the six treatments involving the use of 1.0 per cent Benoclor would be significantly more effective than the 2,4-D controls.

Data in Table III show that mixtures of 2,4-D and Benoclor were

TABLE II

Activation of Hormone-Curvature Responses on Tomato by a Non-Hormone Compound Applied in Combination with 2,4-D and the Relation of These Responses to Herbicidal Activity

Per cent Benoclor 3C	Benoclor 3C and 2,4-D applied as mixtures				Benoclor 3C and 2,4-D applied successively with 24 hours between treatments							
					2,4-D applied first				2,4-D applied last			
	Per cent 2,4-D			Line totals	Per cent 2,4-D			Line totals	Per cent 2,4-D			Line totals
	0.05	0.1	0.2		0.05	0.1	0.2		0.05	0.1	0.2	
Average number of leaves bent downward in 48 hours												
0.0	3	0	4	7	—	—	—	—	—	—	—	—
0.32	3	1	1	5	5	4	1	10	1	2	1	4
1.0	0	1	0	1	4	1	1	6	1	0	1	2
3.2	0	0	1	1	3	2	1	6	0	1	0	1
Totals*	3	2	2	7	12	7	3	22	2	3	2	7
Average number of leaves bent upward in 48 hours												
0.0	1	5	3	9	—	—	—	—	—	—	—	—
0.32	3	5	5	13	1	2	5	8	4	4	5	13
1.0	5	5	5	15	1	4	5	10	5	5	5	15
3.2	6	6	5	17	2	3	5	10	5	4	5	14
Totals*	14	16	15	45	4	9	15	28	14	13	15	42
Degree of stem bending (maximum = 4) in 48 hours												
0.0	2	4	3	9	—	—	—	—	—	—	—	—
0.32	4	4	2	10	3	2	3	8	2	2	2	6
1.0	4	4	4	12	2	3	2	7	1	2	2	5
3.2	4	3	3	10	2	2	3	7	1	2	2	5
Totals*	12	11	9	32	7	7	8	22	4	6	6	16
Average number of days required to kill**												
0.0	11	8	8	27	—	—	—	—	—	—	—	—
0.32	11	9	5	25	10	9	8	27	11	10	9	30
1.0	6	6	5	17	8	7	7	22	8	8	7	23
3.2	7	4	4	15	6	6	6	18	7	5	5	17
Totals*	24	19	14	57	24	22	21	67	26	23	21	70

* Exclusive of 2,4-D controls.
** Data from Table I.

more effective than either component on volunteer seedlings of grasses, smartweed, and purslane. When used separately, 2,4-D was relatively more effective than Benoclor on purslane and smartweed, but less effective on grasses. Solutions containing Benoclor were highly effective for wetting grasses. Presumably those adjuvants which are most toxic to the species of weeds in question would probably be the ones most suitable for use in practice in combination with 2,4-D.

TABLE III

EFFECTIVENESS OF MIXTURES OF 2,4-D AND BENOCLOR 3C AS COMPARED WITH EACH COMPONENT FOR KILLING WEED SEEDLINGS. EXPRESSED AS THE AVERAGE PERCENTAGE KILLED AFTER 7 AND 14 DAYS RESPECTIVELY

Per cent		Kind of seedling							
		Crab grass		Other grasses*		Smartweed		Purslane	
2,4-D	Benoclor 3C	7 Days	14 Days	7 Days	14 Days	7 Days	14 Days	7 Days	14 Days
0.1	—	0	0	0	43	54	89	96	100
—	3.2	59	59	43	52	0	0	48	65
—	10.0	100	100	100	96	45	36	85	93
0.1	3.2	68	86	0	57	52	88	85	100
0.1	10.0	100	100	100	95	96	99	97	100

* Mostly foxtail.

2,4-D and other preparations. Other preparations and compounds gave results similar to those obtained with Benoclor (Fig. 3 and Tables IV and V). In one experiment each of the substances listed in Table IV was used in combination with 0.05 per cent 2,4-D. The effectiveness of these two-substance mixtures ranged from greater than that for the 0.2 per cent 2,4-D control in 1 per cent Tween to little or no increase in activity over that of a water solution of 0.05 per cent 2,4-D. Since the percentages of the preparations are not known to be optimal in all cases, direct comparisons between the different classes of compounds are not justified. However, on the basis of relative differences, the contact herbicides were more effective than the spreaders and solubilizers. Mixtures containing 0.1 per cent α-naphthaleneacetic, β-naphthoxyacetic, or 2,3,5-triiodobenzoic acid were ineffective as activators in this experiment, but in other tests higher concentrations of the first two mentioned hormones (0.2 to 1.0 per cent) were effective.

Non-lethal concentrations of five contact herbicides (listed in Table IV) which by themselves caused slight injury to tomato foliage, generally constituted the minimum concentrations that would function as activators of 2,4-D for killing the test plants. Increasing the concentration of the activator increased the herbicidal activity of the mixture, until a lethal con-

TABLE IV

RELATIVE EFFECTIVENESS OF VARIOUS ADJUVANTS USED AS ACTIVATORS OF 2,4-D FOR KILLING TOMATO

Compound or preparation used as activator	Per cent of activator	No. days required to kill
Used in combination with 0.05 per cent 2,4-D*		
Contact herbicides		
Ammonium thiocyanate	1.0	4
Hammond's Weed Killer	0.2	4
Sodium chloride	5.0	5
Ammate	2.0	7
Diallyl maleate	0.1	8
Spreader, solubilizer, or emulsifier		
Aromatic hydrocarbon oil	1.0	12
Polyethylene glycol 400 monooleate	1.0	13
Penetrol	1.0	13
Vatsol OT	1.0	15
Tween #20	5.0	13
Tween #20	2.0	14
Tween #20	1.0	15
Sodium bicarbonate	1.0	12
Sodium hydroxide	0.1	17
Hormones		
α-Naphthaleneacetic acid (Na salt)	0.1	16
2,3,5-Triiodobenzoic acid (Na salt)	0.1	14
β-Naphthoxyacetic acid	0.1	14
Other 2,4-D* controls		
0.2 per cent 2,4-D in 1 per cent Tween #20		9
0.1 per cent 2,4-D in 1 per cent Tween #20		13
0.05 per cent 2,4-D in water		19

* Sodium salt of 2,4-D made from a purified acid (m.p. 140° C,).

centration of the activator was reached which by itself killed the test plants as quickly as the mixtures, that is, in 3 days or less. Results obtained with ammonium thiocyanate, sodium chloride, and Benoclor are shown in Figure 3. In this case Tween was added to the 2,4-D control solutions (upper row) but not to the mixtures which contained 2,4-D and one of the three activators (lower three rows). Killing effectiveness of the mixtures containing 0.1 per cent 2,4-D and the highest concentration of the activator was equivalent to the 0.2 per cent 2,4-D-Tween control solution (Fig. 3). The average number of days required to kill three plants was as follows according to the activator and in the same order from top to bottom as the treatments indicated in Figure 3 for the plant on the right: 0.2 per cent 2,4-D in Tween, 8.3 days; 0.32 ammonium thiocyanate, 7.0 days; 3.2 per cent Benoclor 7.7 days; and 3.2 per cent sodium chloride, 8.7 days. These values are to be contrasted with 9.7 days for 0.1 per cent 2,4-D in Tween and 15.3

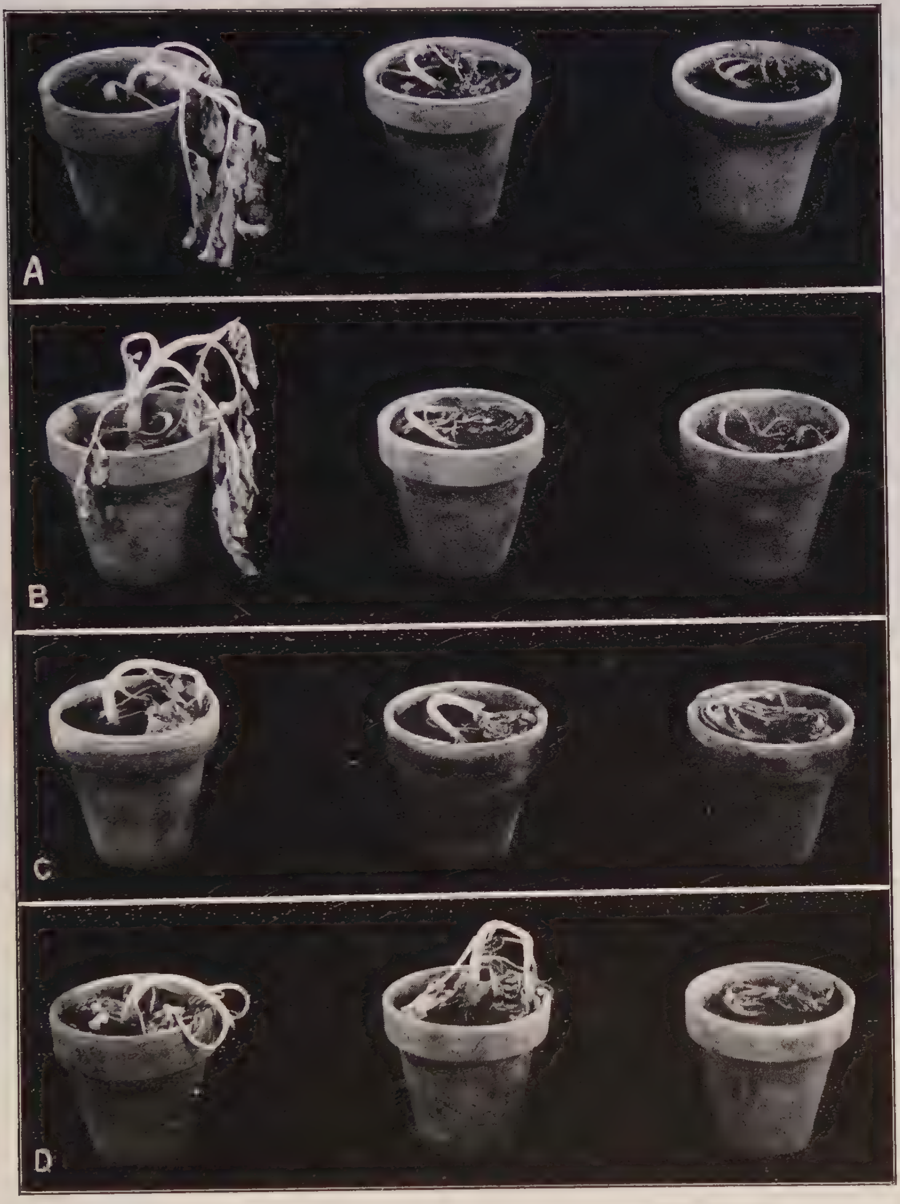

FIGURE 3. Response of Bonny Best tomato 9 days after spray treatment with 2,4-D sodium salt in 1 per cent Tween (A) as compared with mixtures of 0.1 per cent 2,4-D sodium salt and sub-lethal concentrations of a non-hormone toxicant in water solution (B, C, and D). Concentration of 2,4-D in A (L. to R.): 0.05, 0.1, and 0.2 per cent. Concentration of toxicant used in mixtures (L. to R.): B, 0.032, 0.1, and 0.32 per cent ammonium thiocyanate; C, 0.32, 1.0, and 3.2 per cent Benoclor; and D, 0.32, 1.0, and 3.2 per cent sodium chloride.

days for 0.1 per cent 2,4-D in water. These results show that the highest concentrations of the activators were more effective than 1 per cent Tween when used in mixtures with 0.1 per cent 2,4-D. The effectiveness of these same activators in similar mixtures containing 1 per cent Tween, in other tests, was proportionally greater than the 2,4-D-Tween control. Thus the action of these toxicant types of activators was independent of and additional to that obtained with a spreader solubilizer such as Tween.

Results with mixtures of diallyl maleate and 2,4-D furnish additional evidence of activation by a contact herbicide. Data in Table V show that mixtures containing lethal as well as non-lethal concentrations of diallyl maleate caused quicker killing than either component of the mixture. When the concentration of the activator (diallyl maleate) was high enough to kill tomato plants in 2 or 3 days when used alone, no additional killing effects were obtained in mixtures with 2,4-D. On the other hand, there were instances where non-lethal concentrations of activators decreased the killing activity when used in mixtures with 2,4-D. This occurred in some of the tests with Ammate, Hammond's Weed Killer, and 2,3,5-triiodobenzoic

TABLE V

Effectiveness of Mixtures of 2,4-D and Diallyl Maleate* as Compared with the Individual Components in Causing Upward Bending of Tomato Leaves and Death of the Plants

Per cent diallyl maleate	Per cent 2,4-D				Line totals**
	0.0	0.05	0.1	0.2	
Number of leaves bent upward (2 plants)					
0.0	—	0	1	2	3
0.032	0	6	5	6	17
0.1	0	0	5	5	10
0.32	0	5	5	4	14
1.0	0	2	2	1	5
Totals	—	13	18	18	
Average number of days required to kill					
0.0	—	10.0	9.5	7.5	27.0
0.032	†	5.5	4.5	4.5	14.5
0.10	†	5.0	5.0	4.0	14.0
0.32	5.5	5.0	3.5	3.5	12.0
1.0	2.5	2.5	3.5	3.5	9.5
Totals	—	28.0	26.0	23.0	

* Diallyl maleate was not completely soluble in any of the mixtures, agitation of the solution being necessary at the time of spraying.

** Exclusive of diallyl maleate controls.

† Not killed.

acid. There appears to be no satisfactory explanation for this type of interference with the action of 2,4-D by non-lethal concentrations of the activator when higher concentrations of the same activators induced additive effects.

Pre-killing toxic effects. Stem collapse occurred after leaf wilting when the plants were sprayed with 0.05 to 0.2 per cent 2,4-D (Table I). When sprayed with 0.32 to 3.2 per cent Benoclor, stem collapse occurred previous to or simultaneously with leaf wilting. Of the three-combination treatments only one responded like 2,4-D with respect to the relative time of stem collapse, namely, the successive treatment in which 2,4-D was applied first. Thus Benoclor exerted a predominating influence in causing stem collapse and leaf wilting as in the case of killing (line totals vs. column totals, Table I), the two successive treatments being less effective than the mixtures.

HORMONE-CURVATURE RESPONSE

2,4-D and Benoclor. During the course of the present series of tests it was observed that combination treatments which caused quicker killing of tomato test plants also induced responses characteristic of higher concentrations of 2,4-D than were used in the combination treatments. The changes in the rate, degree, and direction of leaf bending which result from increasing the concentration of the applied hormone were described previously for tomato, sunflower, cosmos, marigold, and tobacco (11). These changes may be summarized as follows with special reference to to the present tests. The rate and degree of arched downward bending of tomato stems increased with increasing concentrations of 2,4-D up to a maximum stage in which the curvature resembled a ∩ shape. Maximum bending was generally caused by 0.1 to 0.2 per cent spray solutions of 2,4-D (Fig. 2 A) At still higher concentrations (e.g. 0.4 per cent) the rate and degree of stem bending generally decreased. The rate and degree of leaf bending were also functions of the concentration of applied hormone. In the case of leaf bending, increasing the concentration of 2,4-D changed the direction of bending from downward to upward with reference to the stem tip, and this change in direction was always associated with pronounced downward bending of stems. Examples of downward and upward leaf bending on the tomato are shown in Figure 4 A.

The fact that the curvature responses just described reached a maximum with increasing concentrations of 2,4-D and then decreased makes it difficult to correlate initial bending responses at any given time with the concentration of 2,4-D, since the rate at which the responses occur also varies with the concentration of the applied hormone. If data are recorded at several intervals during the 48-hour period following treatment, changes in curvature can be detected which are not reflected in the data recorded at the end of 48 hours (Table VI). Consequently the differences in curvature resulting from applied 2,4-D are more readily accounted for on the basis of a concentration effect by data in Table V than by data in Table II. In addition to the curvature responses just described, increasing concentrations of 2,4-D induced increasing degrees of proliferation and swelling of stems up to a maximum and thereafter higher concentrations of the hormone caused less proliferation and swelling. There was relatively little swelling or proliferation of stems on plants treated with 0.2 to 0.4 per cent 2,4-D. Thus, slight curvature responses associated with little or no stem swelling after several days would anticipate a relatively quick killing of test plants, but if the curvatures were associated with noticeable stem swelling, the results would anticipate a relatively slow killing effect.

TABLE VI

ACTIVATION OF 2,4-D BY BENOCLOR 3C FOR INDUCING CURVATURE RESPONSES ON TOMATO

Per cent Benoclor 3C	Time after treatment (hours)	Downward leaf bending*				Upward leaf bending*				Stem bending (maximum = 4)			
		Per cent 2,4-D			Line totals	Per cent 2,4-D			Line totals	Per cent 2,4-D			Line totals
		0.05	0.1	0.2		0.05	0.1	0.2		0.05	0.1	0.2	
0.0	4	9	7	9	25	0	2	0	2	0	1	0	1
	6	9	6	5	20	0	3	4	7	0	0	1	1
	24	9	9	3	21	0	0	5	5	1	2	3	6
	48	8	9	1	18	1	0	8	9	—	—	—	—
	Totals	35	31	18	84	1	5	17	23	1	3	4	8
1.0	4	3	0	0	3	6	9	9	24	2	3	2	7
	6	1	0	0	1	6	9	9	24	2	3	1	6
	24	2	0	1	3	5	8	8	21	3	4	2	9
	48	0	0	0	0	9	9	9	27	—	—	—	—
	Totals	6	0	1	7	26	35	35	96	7	10	5	22
3.2	4	0	2	5	7	9	7	4	20	3	2	1	6
	6	0	1	3	4	9	8	5	22	3	2	1	6
	24	0	0	0	0	8	7	7	22	3	4	2	9
	48	0	0	0	0	9	8	9	26	—	—	—	—
	Totals	0	3	8	11	35	30	25	90	9	8	4	21

* Expressed as the number of leaves responding on three plants with a maximum value of 9.

The curvature and swelling responses induced by 2,4-D were induced also by combination treatments with 2,4-D and Benoclor (Tables II and VI). Notwithstanding that Benoclor is a non-hormone preparation which does not induce curvatures on tomato, it functioned as an activator in combination with 2,4-D in causing more pronounced curvature responses than were induced by 2,4-D alone. Unlike tomato, the leaves of purslane bent upward when treated with 3.2 per cent Benoclor and like tomato, the leaves of purslane bent downward when treated with 0.1 per cent 2,4-D. Mixtures of these two preparations hastened and accentuated upward leaf bending of purslane. In this instance 2,4-D appeared to function as an activator of Benoclor.

2,4-D and other preparations. Activation of 2,4-D was obtained with other contact herbicides. Spreaders and spreader solubilizers also func-

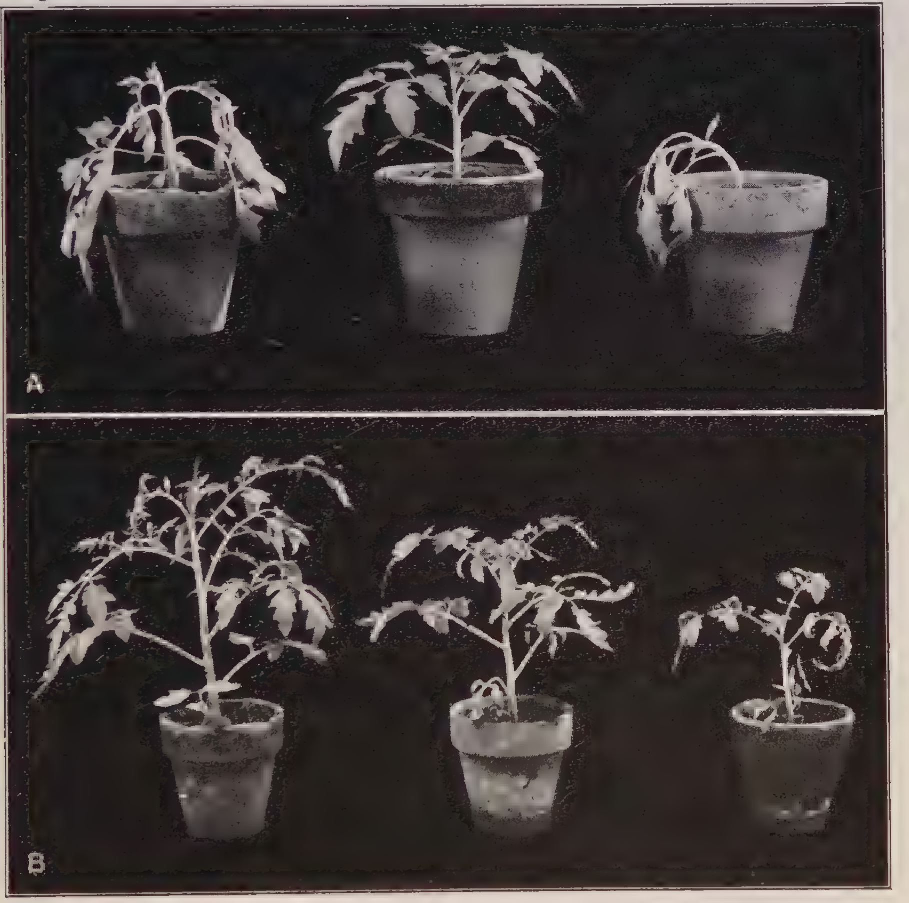

FIGURE 4. Response of Bonny Best tomato to spray treatment with mixtures of 2,4-D and a non-hormone toxicant as compared with the response to the individual components used alone at the same concentration as in the mixture. A. Response after 24 hours (L. to R.): 0.05 per cent 2,4-D, 0.032 per cent diallyl maleate, and mixture of the two. B. Response after 14 days (L. to R.): 0.0001 per cent 2,4-D, 0.32 per cent ammonium thiocyanate, and mixtures of the two.

tioned as activators of 2,4-D for the hormone-curvature responses. The greater effectiveness of hormone preparations containing a Carbowax carrier as compared with water solutions of the same hormones was previously reported (12, p. 332). The following compounds or preparations functioned as activators much the same as Benoclor: Tween, an aromatic oil used as a carrier for hormone esters, sodium bicarbonate, diallyl maleate, ammonium thiocyanate, polyethylene glycol 400 monooleate, DuPont's Ammate, Vatsol OT, and Hammond's Weed Killer. The use of 2,4-D in combination with other hormones (α-naphthaleneacetic, β-naphthoxyacetic, and 2,3,5-triiodobenzoic acids) also caused more pronounced curvature responses but no attempt was made in these tests to determine whether the effects were greater than additive. An example of the more pronounced curvatures induced by a mixture of 2,4-D and diallyl maleate as compared with the effectiveness of 2,4-D alone is shown in Table V and is illustrated in Figure 4.

RELATION BETWEEN CURVATURE RESPONSES AND KILLING

Tomato plants which exhibited pronounced upward bending of most or all leaves during the first 48 hours died sooner than plants exhibiting little or no upward leaf bending. For example, the plant on the right in Figure 4 A died 5 days sooner than the plant on the left (same figure). Data in Table II show that 21 of the 27 combination treatments with Benoclor and 2,4-D caused maximum or near maximum upward leaf bending, yet the time of death of these plants varied from 4 to 9 days. The other six treatments which caused three leaves or less to bend upward induced death of the plants in 7 to 11 days. From these results it follows that it is not possible to determine which treatments are most effective on the basis of the initial curvature responses unless the dosage ratio is adjusted so as to give near minimum curvature responses with the lowest concentrations, intermediate responses with intermediate concentrations, and maximum responses with the highest concentration. These conditions were met in the present tests only in the case of the successive treatment in which 2,4-D was added first (Table II). In this case the increasing number of leaves bending upward resulted from increasing concentrations of 2,4-D at each concentration of Benoclor. There was a corresponding decrease in the time of death with increasing concentrations of 2,4-D. This was the only one of the three combination treatments in which 2,4-D exerted a predominating influence with each successive increase in concentration. As mentioned in the last part of the preceding section, it is possible under certain conditions to anticipate relatively slow or quick killing of test plants on the basis of initial curvature responses being associated respectively with marked stem swelling or practically no stem swelling.

Relation of 2,4-D to activation. In the present series of tests any adjuvant which caused quicker killing of tomato plants when used in combination with 2,4-D has been regarded as an activator provided the mixture was more effective than either 2,4-D or the adjuvant. Considering the different classes of substances which increased the herbicidal activity of 2,4-D, it would appear that these activators functioned in one or more of the following ways: (a) as a wetting agent whereby the maximum surface of the plant tissue was contacted by the hormone mixture; (b) as a solubilizer or emulsifier for maintaining solubility of 2,4-D in the solution, on the surface of the tissue, and possibly inside the tissue; (c) as a penetrant in aiding the entrance of 2,4-D without noticeable damage to the tissue; (d) as a toxicant which alters the permeability of the tissue for 2,4-D and may involve initial killing of tips and margins of some leaflets; and (e) differential effects whereby the activator and 2,4-D may act independently or jointly because of differences in their capacity to be transported or because the activator may affect different tissues or the same tissues to a different degree as compared with 2,4-D.

When the spreader solubilizer such as Tween caused a substantial increase in the herbicidal activity of 2,4-D, an additional substantial increase in activity was obtained by the addition of another class of activator such as a contact herbicide. Since Benoclor 3 (non-emulsified form) added to 1 per cent Tween functioned much like Benoclor 3C there are cases in which the carrier of 2,4-D can have several adjuvants, each of which may function as an activator of 2,4-D. No attempt was made in these tests to determine whether the combined effect of several adjuvants was proportional to effects obtained separately with each one.

Under certain conditions it is possible that either 2,4-D or an adjuvant such as a contact herbicide may function as an activator in mixtures. For example, when a non-lethal concentration of a contact herbicide which caused slight killing of tomato foliage was combined with 0.1 per cent 2,4-D, the mixture caused considerably more initial killing of foliage and killed the plants in a shorter time. In this case, 2,4-D might be regarded as the activator in bringing about a greater initial killing. But since 2,4-D in the range 0.2 to 0.4 per cent also causes initial killing of tomato foliage, the contact herbicide might be regarded as the activator of 2,4-D in making the mixture equivalent to a higher concentration of 2,4-D. The same explanation might be used also for the final toxic effects whereby all plants were killed more rapidly by the above-mentioned mixture. Since 0.1 per cent 2,4-D killed tomato plants in about 8 days and the mixture killed them in a shorter time, it would appear that a non-lethal concentration of the contact herbicide functioned as an activator in the mixture. But since a higher concentration (two to four times) of the contact herbicide was lethal to tomato, it might be said that 2,4-D activated the contact herbicide in causing the latter to function as a higher concentration in the mixture. The inadequacy of these explanations serves to show the need for more factual information relating to the action of one or more adjuvants in a hormone-herbicidal mixture.

In one series of tests it appeared that only one of the two active components in a mixture could function as the activator. Mixtures containing very low concentrations of 2,4-D (0.00001 to 0.0001 per cent) and a non-lethal concentration of ammonium thiocyanate (0.32 per cent) caused considerably more killing of tomato foliage and a much greater delay in growth than either of the components used alone. When used alone at such low concentrations, 2,4-D actually stimulated the growth of tomato by increasing the rate of stem elongation. Consequently, the greater toxic effects of the mixtures (Fig. 4 B) appear to result from the function of 2, 4-D as an activator of ammonium thiocyanate and not conversely. In these particular tests there was no significant increase in the degree of modification or the number of leaves modified by the mixtures as compared with 2,4-D used alone.

The use of several adjuvants such as those previously mentioned under items (a) to (d), in herbicides, has been reported by others (1, 9). However, up-to-date toxicants such as contact herbicides have not been used in mixtures with 2,4-D in commercial formulations.

Results of the present series of tests indicate that lethal as well as non-lethal concentrations of toxicants such as contact herbicides can be used to advantage in formulations of 2,4-D without inhibiting although possibly reducing the latter's important action as a hormone. As a matter of fact, 2,4-D functions as a toxicant particularly when used at concentrations in excess of 0.1 per cent and consequently kills tissue within a few hours to several days in much the same way as a contact herbicide. For example, in connection with tests recorded in Table I, 0.4 per cent 2,4-D had killed the growing tip and parts of many leaflets at the end of 24 hours, the entire plant being killed in 4 days (footnote**, Table I). Treatments which cause rapid killing of above-ground parts of plants are not necessarily the ones which are most effective in practice for killing roots of perennials. For example, in tests with dandelions (7, p. 491) the percentage regrowth from roots was frequently the highest in plots treated with 0.2 per cent 2,4-D where the percentage top kill was greatest and the rate of killing tops was most rapid. There was less recovery in dandelion plots where killing of tops with a mixture of 0.1 per cent each of 2,4-D and α-naphthaleneacetic acid was slower.

Under certain conditions 2,4-D appears to function as an activator of a toxicant such as ammonium thiocyanate. This occurred in certain tests designed to determine whether a non-hormone compound could activate 2,4-D with respect to modification of tomato leaves which is a hormone response. In these tests 0.0001 per cent 2,4-D caused an increase in stem elongation over water-sprayed controls whereas 0.32 per cent ammonium thiocyanate delayed stem elongation and caused local injury and slight yellowing of leaves. A mixture of the two compounds at the stated concentrations caused a marked delay in growth, and caused more extensive killing of leaf tissue. The appearance of these plants 12 days after treatment is shown in Figure 4 B. Eventually the plant on the right resumed growth and produced modified leaves, but there was no indication of an increase in the degree of modification due to the presence of ammonium thiocyanate in the mixture. Final records with reference to the total numbers of modified leaves are not available at this time.

CONCLUSIONS AND SUMMARY

Mixtures of 2,4-D and certain adjuvants (Benoclor 3C, ammonium thiocyanate, ammonium sulfamate, Hammond's Weed Killer, diallyl maleate, sodium bicarbonate, and sodium chloride) were more effective herbicides than any of the individual components used at the same concentration as in the mixture. Adjuvants which functioned as activators of 2,4-D included wetting agents, solubilizers, emulsifiers, penetrants, hormones other than 2,4-D, and toxicants commonly used as contact herbicides. Mixtures containing Tween #20 and one of the contact herbicides in combination with 2,4-D were most effective in killing the test plants.

Mixtures containing lethal and sub-lethal concentrations of contact herbicides increased the activity of the hormone-like preparations with respect to the induction of hormone-curvature responses, initial injury to foliage, and killing of the entire plant. The herbicidal activity of these mixtures increased with increasing concentrations of the contact weed killer up to the point where the mixture was no more effective than the contact weed killer used alone at the same concentration as in the mixture. Equivalent activity in this case meant killing the test plants in 3 days or less. Relatively high concentrations of contact herbicides (1 to 30 per cent) were required to prevent 2,4-D from inducing additive effects.

Considering the different kinds of substances, including sodium chloride, which increased the herbicidal activity of 2,4-D, it seems apparent that they function mainly as general toxicants in altering the permeability of the tissue and thus hastening the penetration of 2,4-D. Since many hormones, including 2,4-D, also act as toxicants when used at relatively high concentrations, they are not excluded from the types of activators which may function as toxicants.

Some of the toxicants are probably transported in plants more effectively than others and consequently in addition to exerting its own toxic effects, the activator might aid in extending the penetration of 2,4-D inside the plant as well as through the epidermal layer. On this basis it would be possible for a toxicant type of activator to act independently as well as jointly with 2,4-D. Particularly in the case of mixtures containing more than one hormone, different tissues might be affected preferentially by different hormones.

When mixtures containing a toxicant type of activator caused more pronounced curvature responses than were obtained with the same concentration of 2,4-D used alone, the responses were the same as those induced with a higher concentration of 2,4-D. These more pronounced curvature responses which consisted of upward leaf bending, pronounced downward stem bending, and little stem swelling, were indicative of a relatively rapid killing of the test plants (8 days or less). Lower concentrations of 2,4-D (less than 0.1 per cent) which caused mainly downward leaf bending, less pronounced stem bending, and considerable stem swelling were indicative of a relatively slow killing (10 days or more). Since hormone-induced curvature responses are the result of differential growth, such responses did not occur when the concentration of applied hormone was high enough to prevent growth. Leaves of tomato were inhibited by lower concentrations than stems.

Mixtures containing 0.32 per cent ammonium thiocyanate and 0.00001 to 0.0001 per cent 2,4-D caused greater injury to tomato foliage and a greater delay in growth of the plant than 0.32 per cent ammonium thiocyanate used alone. However, there was no significant increase in the modification of leaves caused by mixtures as compared with 2,4-D used alone. Such low concentrations of 2,4-D were neither toxic nor inhibitive to tomato. On the contrary, they stimulated tomato by increasing the rate of stem elongation. In these tests 2,4-D appeared to function as the activator and not ammonium thiocyanate, although in other tests ammonium thiocyanate activated lethal concentrations of 2,4-D (0.05 to 0.2 per cent).

The various substances used as activators in the present tests probably represent only a relatively small number of those which would prove equally or more effective when used in mixtures with 2,4-D or other hormone herbicides. Differences in the herbicidal activity of different 2,4-D formulations are probably explainable to a large extent on the basis that the adjuvants aid the penetration of 2,4-D regardless of whether it is present as an acid, ester, or salt.

It is believed that more effective general-purpose herbicides than those used to date are likely to contain more than one hormone, at least one toxicant, and additional adjuvants which are effective as wetting agents, solubilizers, penetrants, emulsifiers, and stickers.

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DEVELOPMENT OF COPPER-ZINC-CHROMATE COMPLEXES AS POTATO FUNGICIDES[1,2,3]

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INTRODUCTION

The fungitoxic properties of chromium and compounds of other metals with chromium have been known for some time (3, 5). However, salts of chromium have never been practical as fungicides for the control of foliage diseases because the soluble chromium salts are phytotoxic. Hence, the use of chromates in the past has been limited to wood and fabric impregnation.

After the development of the "fixed" coppers as fungicides, the attention of research workers was turned toward organic compounds, and the inorganic fungicides have received relatively little attention in recent years. Certain of the authors tested (1) various compositions containing chromates since chromium compounds were known to be fungicidal. All of those tested were ineffective as foliage fungicides (10) because the ones that controlled diseases in the greenhouse were phytotoxic. Special chromates were then synthesized, some of which were effective foliage fungicides and others were not. Those classed as effective were fungicidal, not phytotoxic at effective concentrations, and resistant to weathering on foliage. Some of the materials prepared showed a fungitoxic synergistic effect of chromium with certain other metals. From chemical analyses of the chromates it was found that those which were fungitoxic contained chromium in the hexavalent state, and it is in this valence state that chromium occurs in the the complexes described in this paper.

SOLUBILITY RELATIONSHIPS

From chemical anlyses of the suspensions of these chromate complexes at varying percentage concentrations, the fact emerged that the solubility

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[2] This research was sponsored by Carbide and Carbon Chemicals Corp., New York 17, N. Y. and conducted in part at Boyce Thompson Institute for Plant Research, Inc., Yonkers 3, N. Y., at the Pennsylvania State College, State College, Penna., and at the Linde Air Products Company, Tonawanda, N. Y.

[3] This article was preprinted August 20, 1948.

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of the component metals in the complex increased with the concentration of the suspension (Table I). Further, the molar ratios of the components of the complex in solution differed from the original solid and varied with the amount of dilution (Table II).

TABLE I

RELATION OF SOLUBILITY TO SUSPENSION CONCENTRATION

Complex	Solubility at per cent suspension concn. of						
	G. Cr^{+6}					G. Zn^{+2}	
	0.1	1.0	10	30	50	1	10
Zn-Cr	—	0.0013	0.0066	0.0105	—	0.0014	0.0050
Zn-Fe-Cr	0.0027	0.0151	0.0900	0.1950	0.2600	0.0110	0.0400
Cu-Zn-Cr	—	0.00074	0.00537	—	—	—	—

TABLE II

MOLAR RATIO OF ZINC TO CHROMIUM IN SOLID AND SOLUTION

Complex	Molar ratio zinc to chromium		
	In orig. solid	In solution at per cent suspension concn. of	
		1	10
Zn-Cr	4.04	0.86	0.60
Zn-Fe-Cr	2.46	0.58	0.35

This indicated that the complex did not go into solution as a unit but that water acted to change its composition. Thus, if complexes of this type were washed during or subsequent to their preparation, one factor determining their composition would be the amount of washing. The action of the water was reversible. The dissolved portion of the complex reformed the solid phase again when the suspension was concentrated to, at most, one-fifth of its original volume by vacuum evaporation of the water at room temperature. This is indicated graphically in Figure 1.

When chromate solubilities were plotted against suspension concentrations on log-log scale, nearly straight lines were obtained. The triangular points on Figure 1 were the solubilities following vacuum concentration. If the soluble chromium were merely being concentrated by evaporation, its solubility would increase proportionately to the suspension concentration, or along the dotted lines in Figure 1. Instead, the chromate solubility returned to approximately its original value, indicating that the dissolved chromium re-entered the solid complex.

These deviations from the normal behavior of suspensions indicated the need for a standard per cent suspension for solubility comparisons of the complexes. A standard suspension of 1 gram of solid in 100 ml. of distilled water was selected, since this represented approximately 8 lb./100 gallons or about the maximum concentration anticipated for field use of any complexes developed. Bioassays were made to discover the range of chromate solubilities that would be fungitoxically effective and that would not, at the same time, be phytotoxic to plants in foliage. Test-tube dilution spore germination tests (1, 2) and greenhouse disease control and phytotoxicity tests (7, 10, 11) were made of complexes covering a wide range of solubilities. The greenhouse disease control tests were made on tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) and the phy-

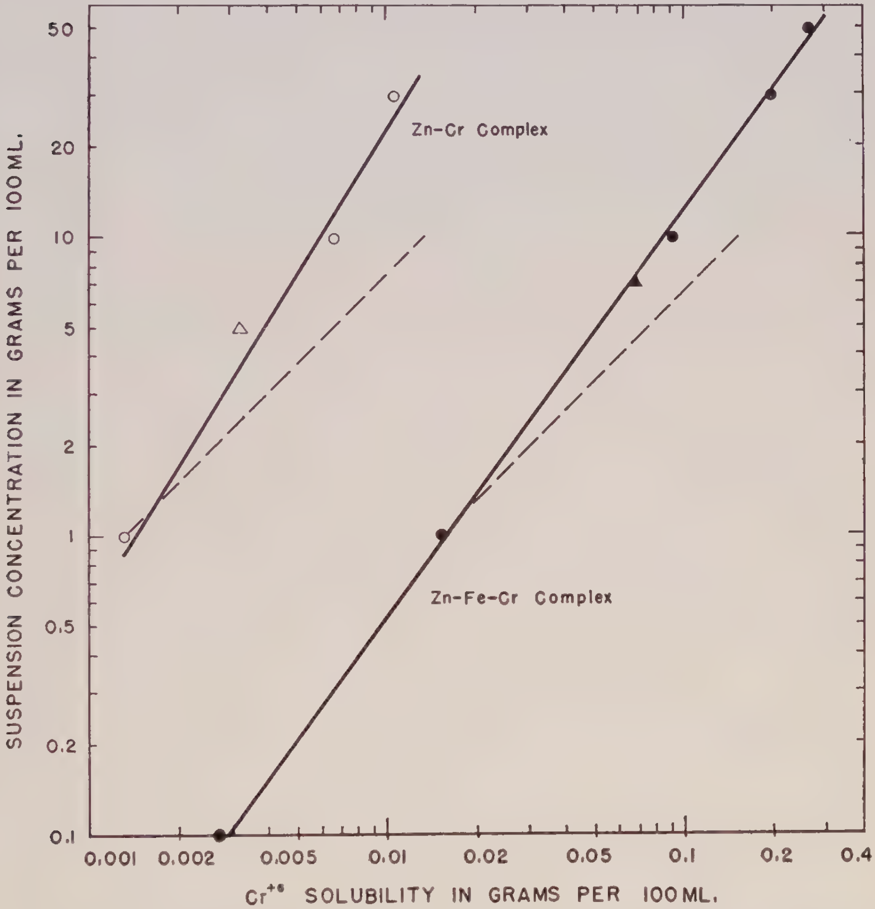

FIGURE 1. Chromate solubility as a function of suspension concentration. Triangle—actual chromium solubility following concentration by evaporation. Dotted line—expected chromium solubility if chromium were merely being concentrated by evaporation.

totoxicity tests on bean (*Phaseolus vulgaris* L. var. Tendergreen), buckwheat (*Fagopyrum esculentum* Moench.), tobacco (*Nicotiana tabacum* L. var. Turkish), and tomato. The results from several of the complexes tested are given in Table III. Further bioassays established that Cr^{+6} solubility had to be above 0.0001 g./100 ml. at the standard 1 g./100 ml. suspension to be fungitoxic and below 0.03 g./100 ml. to be without serious phytotoxicity to plants in foliage (Table IV).

TABLE III

RELATION OF CHROMATE SOLUBILITY TO TOXICITY

Complex	Solubility, g. Cr^{+6}/100 ml.	Av. concn. in p.p.m. of complex for ED50* against spores**	Av. per cent greenhouse disease at 0.2% spray†	Av. phytotoxicity of 1% spray‡
Pb-Cr	0.000001	1000	70	None
Ca-Cr	0.5000	3	0	Severe
Cu-Cr	0.0005	1	0	None
Cu-Zn-Cr	0.0004	2	0	None

* ED50, effective dose for 50 per cent spore inhibition (6, footnote 8).
** *Sclerotinia fructicola* (Wint.) Rehm.; *Alternaria oleracea* Milbraith.
† Early Blight and Late Blight of tomato.
‡ To bean, buckwheat, tobacco, and tomato plants.

TABLE IV

DETERMINATION OF CHROMATE SOLUBILITY LIMITS FOR FUNGITOXIC EFFECTIVENESS AND PHYTOTOXIC SAFETY

Complex	Solubility, g. Cr^{+6}/100 ml.	ED50 in p.p.m.*		Per cent disease after 0.2% spray**		Phytotoxicity of 1% spray†
		S.f.	*A.o.*	E.B.	L.B.	
Pb-Cr	0.000001	1750	890	100	100	A
Ba-Cr	0.00007	>1000	>1000	76	96	A
Cu-Cr	0.0001	0.8	1.7	15	5	A
Cu-Cr	0.004	2.1	0.9	2	3	A
Cu-Cr	0.017	1.4	0.7	3	3	B−
Fe-Cr	0.027	2.0	0.5	2	—	C
Zn-Fe-Cr	0.13	1.4	0.2	5	7	D+
Ca-Cr	2.3	0.7	0.6	—	—	E

* See Table III, footnote**.
** E.B. = Early Blight and L.B. = Late Blight of tomato.
† Average phytoxicity to bean, buckwheat, tobacco, and tomato. A = no injury; B = slight injury; C = moderate injury; D = severe injury; E = plant dead.

NATURE OF THE CHROMATE COMPLEXES

Dalton was the first to clearly envision atoms and their relationships when united as chemical compounds, thus offering a rational explanation for the empirical laws of chemical combination. Deviation of the chromate complexes from the normal expected behavior of stoichiometric compounds in suspensions indicated that they fit the general concept of non-Daltonian complexes.

The composition of stoichiometric compounds is determined by the electron-pair covalent bonds formed by the component atoms or by the valency of ions formed by these atoms. Dalton's law of constant proportions is corollary to the nature of these valence forces responsible for such chemical combinations. This general principle is, however, inadequate to explain aberrant types of combination, *viz.* nitrides and carbides of heavy metals, transition metal oxides and sulfides, intermetallic and interstitial compounds. The true structural unit in these cases is the unit crystal cell; the concept of the molecule has no place in the formation of such compounds. The composition of the unit crystal cell is governed primarily by atomic packing and the balancing of ionic charges. A tolerance of composition is possible and obedience to Dalton's law may be only accidental. It is only in exceptional cases (*e.g.* the tungsten bronzes) that the tolerance of variability is other than very small (4). Certain of the chromate complexes are such exceptional cases. Usually the omission of more than a small proportion of one species from the crystal lattice would distort and fracture the crystal structure; such is not the case with the chromate complexes.

The complexes described in this paper do not fit Dalton's concept of combination by constant proportions. They are distinct and reproducible chemical entities having components that are capable of being continuously varied over a wide range of composition. It will be shown later in this paper that the chromate complexes are not simple mixtures.

These chromate complexes do not fit the concept of solid solutions if such a solution is defined as a mixture of two or more chemical substances, the mixture having the same chemical and physical properties in every part and the component chemical substances retaining their properties affected only by dilution. In the chromate complexes, reaction takes place and the properties of the components are often markedly changed.

THE ROLE OF ZINC- AND COPPER-CHROMATE COMPLEXES

Laboratory bioassay clearly indicated the fungitoxic effectiveness of the chromates (Tables III and IV). Field tests on potatoes (*Solanum tuberosum* L.) in 1942 at State College, Pennsylvania, confirmed the ability of copper-chromate complex to control fungus diseases at 1 lb./100 gallons. In spite of adequate disease control, the yield of potatoes was poor partly because of failure to repel insects. In 1943, tests in the same area showed that a basic zinc-chromate complex controlled tip burn, probably by repelling the leafhoppers (*Empoasca* spp.) which initiate the disease (Table V).

The 1944 field test was more comprehensive and sought to confirm the usefulness of the copper-chromate, zinc-chromate, tank mixture of copper-chromate and zinc-chromate, and the copper-zinc-chromate complexes. The plots consisted of four adjacent 100 foot rows, which were replicated four times and randomized but planned so that no two treatments occurred side by side more than once. Eight spray applications were made at 10 to 14 day intervals beginning when the potato sprouts were 4″ to 6″ high. Certified seed of var. Russet Rural potatoes was used. Spraying at 400 lbs. tank pressure was done by a tractor-drawn power sprayer equipped with a 12-nozzle, 4-row, adjustable height row crop spray boom. Approximately 125 gallons of spray were applied at each application.

Fungus diseases in measurable amount did not develop in the course of the experiment, so the test was essentially one of insect control. No insecticides *per se* were applied, making the degree of insect control solely a function of the chromate complexes. A direct measure of the degree of insect control was obtained by mid-season counts of flea beetle (*Epitrix* spp.) feeding holes, and leafhopper nymphs on 25 terminal leaflets selected at random from plants in the two inside rows of each plot.

TABLE V

POTATO FIELD TEST, STATE COLLEGE, PENNSYLVANIA, 1943

Fungicide	Concn., lb./100 gal.	Late Blight estimate*	Tip burn estimate*	Yield, lb./plot
Bordeaux	8–8	14	2	1408
Tribasic**	4	22	19	1314
Zn-Cr	2	31	12	1277
Cu-Cr	1	13	39	1149

* Total estimate of 3 observers for 4 replicates on rating scale of 0–4; low numbers reflect high control.

** Tribasic copper sulfate.

Three weeks after the insect counts were made, an estimate of the amount of tip burn in each plot was obtained by ranking the five terminal leaflets from each of twenty compound leaves selected at random from the two inside rows of each plot. Five weighted classes of the amount of dead tissue on each leaflet were given numerical ratings of 0 to 4. Averages of the tip burn index were significantly correlated with the leafhopper nymph count and provided a more representative measure of the real amount of plant damage, since, when the insect counts were made earlier, the leafhopper population was low and variable.

None of the chromate complexes tested prevented flea beetle feeding. Certain of them did repel leafhoppers and thereby reduced the amount of tip burn, which was the major yield-controlling factor in this experiment. This is indicated in Table VI.

TABLE VI

POTATO FIELD TEST, STATE COLLEGE, PENNSYLVANIA, 1944

Plot No.	Complex	Concn., lb./100 gal.				Tip burn index	Lb./plot, corrected yield*
		Total	CuO	ZnO	CrO_3		
1	Zn-Cr	3	—	2.00	0.60	51	218
2	Zn-Cr+Cu-Cr	1½+½	0.30	1.00	0.43	76	210
3	Zn-Cr	1	—	0.67	0.20	88	198
4	Zn-Cr	⅓	—	0.22	0.07	115	157
5	Cu-Zn-Cr	1	0.56	0.08	0.24	99	195
6	Cu-Zn-Cr	2	0.64	0.64	0.42	70	214
7	Cu-Zn-Cr	2	0.28	1.12	0.34	66	220
8	Cu-Cr	1	0.60	—	0.25	134	167
9	Cu-Cr	½	0.30	—	0.12	140	168
10	Cu-Cr	¼	0.15	—	0.06	138	155

* Dug yields corrected by geographical correction method of Wellman *et al.* (12).

When tip burn indices and yield were plotted against the log of the ZnO dosage (Fig. 2 A and B) treatments fell into two groups giving nearly straight lines. Plots 1, 3, and 4 were zinc-chromate complexes; 5, 6, and 7 were copper-zinc-chromate complexes. Plot 2 was a tank mixture of zinc-chromate complex and copper-chromate complex. In tip burn control it behaved like and was equivalent to zinc-chromate complexes alone. Copper-zinc-chromate complexes were better than zinc-chromate complexes in tip burn control at equivalent ZnO dosages although at high ZnO dosage (1-1/2 lb./100 gals.) the difference disappeared.

Yield, in this experiment, was largely a function of tip burn. Since tip burn was correlated with ZnO dosage and the type of complex, yield was highly correlated with ZnO dosage. Again, the tank-mixed zinc-chromate complex+copper-chromate complex behaved as a zinc-chromate complex. Again, the copper-zinc-chromate complexes were superior to the zinc-chromate complexes at equivalent ZnO dosages. Since convergence of the concentration response curves for yield occurred at ZnO concentrations greater than for tip burn control, Zn must have had an effect on yield beyond tip burn control.

The copper-chromate complex failed to control tip burn and zinc-chromate complexes controlled this adequately. The superiority of the copper-zinc-chromate complexes must have been due not only to the successful control of tip burn disease, but also to the control of such fungus diseases as were not measurably evident, or to zinc stimulation. The zinc-chromate complex and copper-chromate complex mixed together in the tank behaved as a zinc-chromate complex alone. This was confirming evidence that both zinc and copper had to be built into the same complex. Simple mixtures were not as efficient.

Yields of potatoes sprayed with copper-zinc-chromate complexes were

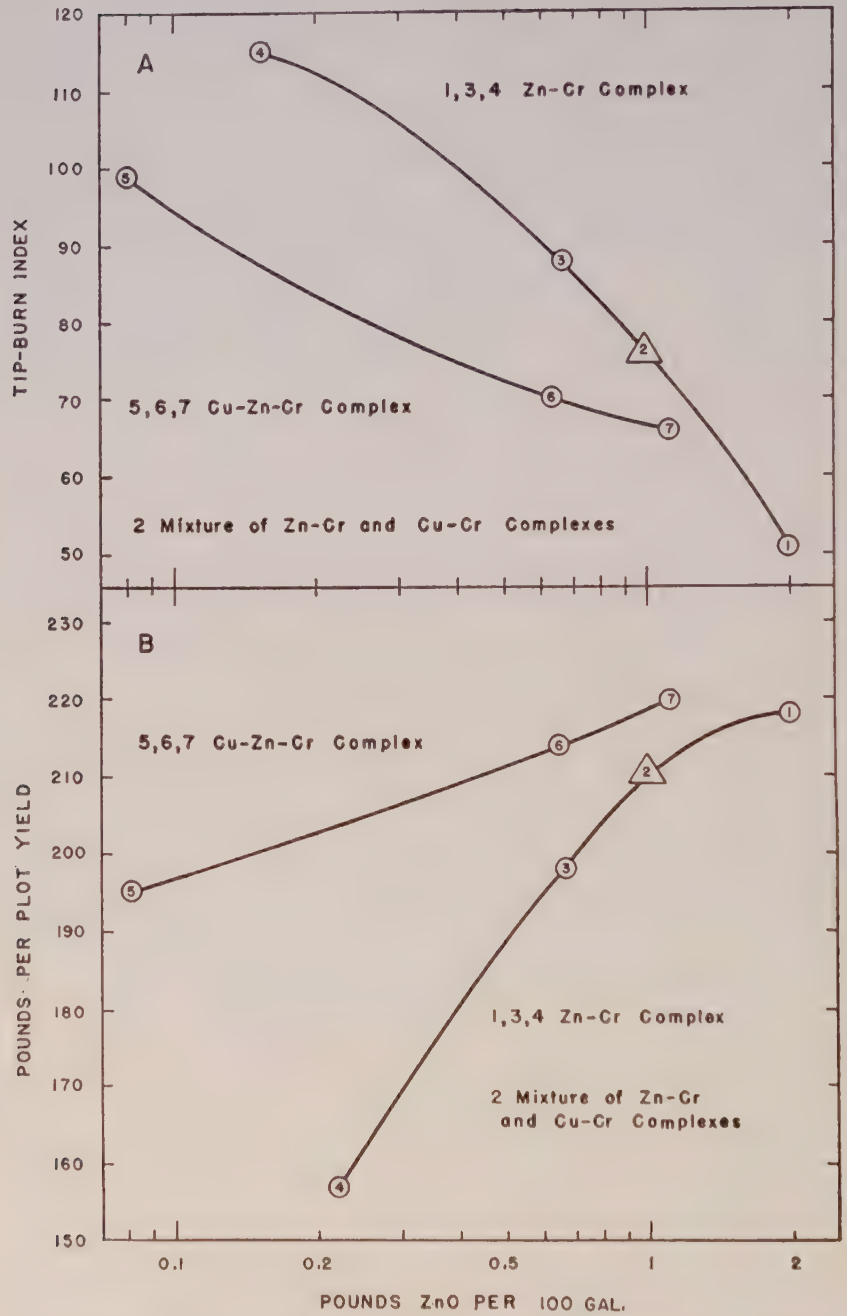

FIGURE 2. A. Tip burn index as a function of ZnO concentration. B. Yield of potatoes as a function of ZnO concentration.

approaching that when Bordeaux mixture was used. By extrapolation, it was calculated that 5.5 lb. of copper-zinc-chromate complex (Plot 7, Table IV) coded 169, containing 3.0 lb. of ZnO equivalent, should equal Bordeaux. In tests conducted at State College in 1945, this was the case (Table VII). Tests in other areas *e.g.*, Florida, Long Island, N. Y., indicated that this low-copper high-zinc-chromate complex, coded 169, was the equivalent of Bordeaux mixture, thus confirming the predictions based on previous tests.

TABLE VII

POTATO FIELD TEST, STATE COLLEGE, PENNSYLVANIA, 1945

Fungicide	Concn., lb./100 gal.	Per cent			Corrected yield, lb./plot
		Flea beetle	Tip burn	Late Blight	
Bordeaux	8–8	12	30	10	217
Cu-Zn-Cr (169)	5½	20	36	19	211
Check	—	100	100	100	78

LSD for corrected yield in lb. per plot 24 at 19:1; 31 at 99:1.

DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] was almost universally adopted for use on potatoes in 1945. It gave practically perfect control of the major yield-reducing potato insects. Thus, the insect repellent properties of the copper-zinc-chromate complexes appeared no longer necessary.

Since the copper-chromate complex in earlier tests had given excellent potato disease control, and since insects could now be so adequately controlled by DDT, this combination was tested. It failed to yield satisfactorily. This was further indication that zinc functioned in the complex in some other way than as an aid to tip burn control. Disease control and yields were better with copper-zinc-chromate plus DDT than with copper-chromate plus DDT at comparable copper and chromium dosages.

THE COPPER-ZINC-CHROMATE COMPLEX SERIES

The 1944 tests showed that the copper-zinc-chromate complexes were superior to zinc-chromate complexes in insect repellency and consequent tip burn control. Here the key element was zinc. Tests through 1946 showed that the copper-zinc-chromate complexes were superior to copper-chromate complex in fungus disease control. Here the key elements were copper and chromium. This dual synergism led to a further study of the copper-zinc-chromate complexes.

X-ray diffraction studies indicated that the copper-zinc-chromate complexes yielded a definable spectrum. Data were read from spectrometer charts obtained from a North American Philips X-ray Spec-

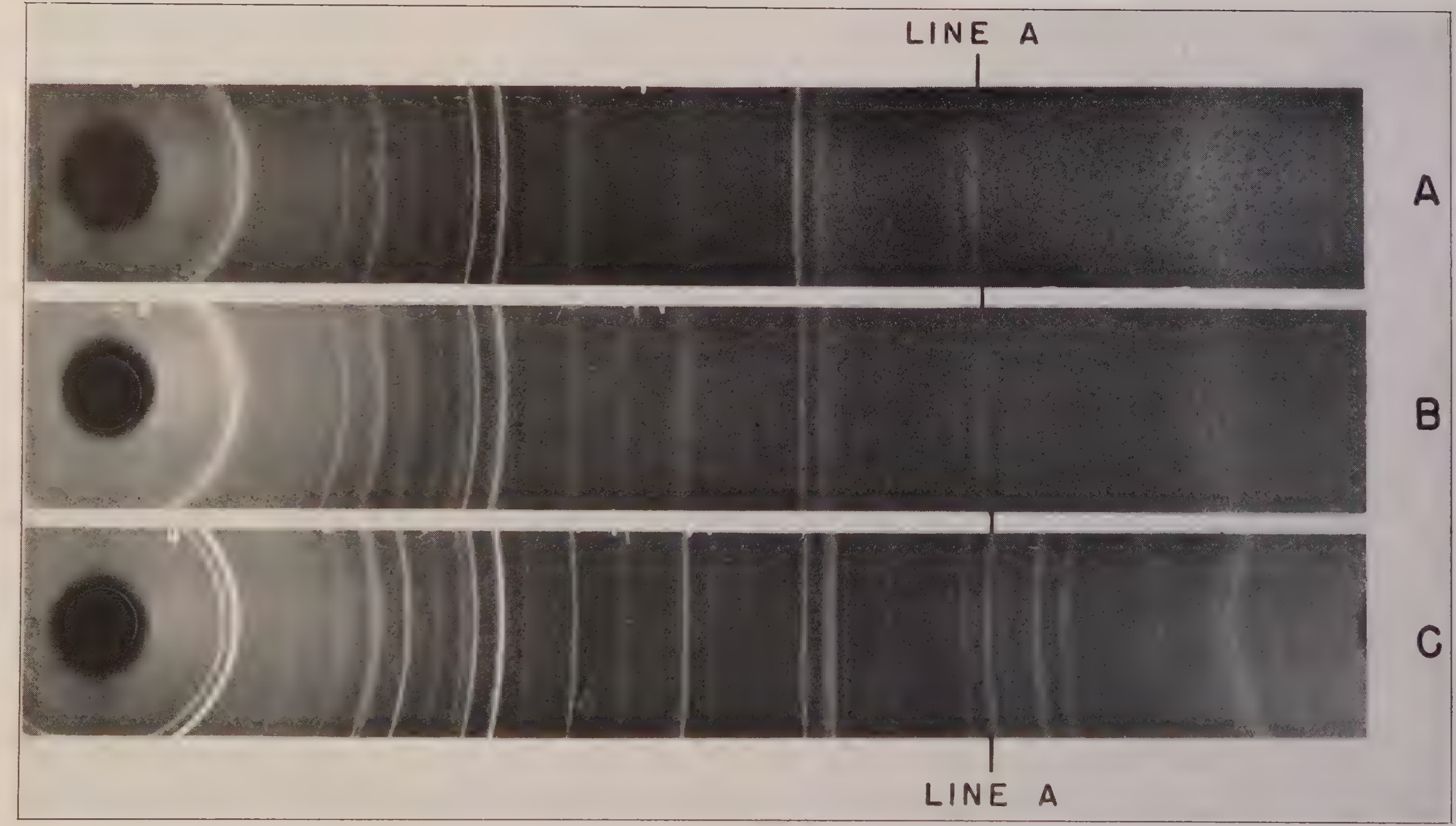

FIGURE 3. Typical X-ray diffraction patterns of copper-zinc-chromate complexes: A, basic zinc-chromate (181); B, low-copper high-zinc-chromate (169); C, medium high-copper low-zinc-chromate (658).

trometer employing copper radiation, a Geiger counter, and a Brown recording potentiometer. A chromium target tube is preferred for X-ray analysis of chromates due to the relatively strong fluorescence of chromium in radiation from a copper target tube. The X-ray photographs shown in Figure 3 were obtained by employing a camera of 57.3 mm. radius (1 mm. = 2° Bragg angle, θ) with a chromium target tube in standard diffraction equipment.

The X-ray diffraction pattern was generally similar for all copper-zinc-chromate complexes studied regardless of the proportions of the components, and was roughly equivalent to that of basic zinc chromate. In contrast to this, copper-chromate complexes had an entirely different pattern. The only result of varying the proportions of the components (within broad limits) by adding copper or partially replacing zinc with copper in copper-zinc-chromate complexes was to change the interplanar distances (*d*) in the crystal lattice as shown in the X-ray diffraction patterns. This is indicated in Table VIII. These data were obtained from the photographs of the X-ray diffraction patterns reproducd as Figure 3. No new lines appeared and the lattice remained unchanged except for increasing definition of the lines due to resonance and to the contraction of the lattice which appears in Figure 3 as a gradual expansion of the X-ray diffraction pattern because the sine of the angle of diffraction is inversely proportional to the interplanar distances.

TABLE VIII

PARTIAL X-RAY PATTERNS OF COPPER-ZINC-CHROMATE COMPLEXES (CHROMIUM TARGET)

Chromate Code No.	Approximate atomic ratio			X-ray pattern		
	Zn	Cu	Cr	Line	Bragg angle, θ	Interplanar distance, *d*
181	4	0	1	A B	58.85 73.75	1.3360 1.1910
169	4	1	1.5	A	59.40	1.3285
658	2	3	1.2	A B	60.10 76.72	1.3190 1.1749

When zinc replacement by copper was continued beyond the ratio of 0.8 Zn to 4.2 Cu, to 1.375 Cr, the diffraction pattern changed as new lines appeared superimposed on the basic zinc-chromate pattern.

Not only could the relative and actual zinc and copper in the complex be varied over a wide range of composition, but the relative and actual amount of chromium could likewise be varied. The higher the copper to zinc ratio, the more chromium could be absorbed into the lattice of the complex. If too much chromium were used, the unreacted part could be detected by high Cr^{+6} solubility; if too little were used, the other components of the complex appeared in X-ray diffraction patterns as lines of unreacted zinc or copper not common to the pattern of the copper-zinc-chromate complex. Thus, the extent of reaction was followed by the disappearance of the X-ray patterns of the reactants. In completely reacted products, the components were apparently absorbed into the crystal lattice of the complex leaving no trace of their presence except for increasing sharpness of lines and the contraction of the interplanar distances. The mere use of the reactants in the correct proportions did not assure complete reaction. Field tests of incompletely reacted products showed that efficient disease control and high yield were functions of the degree of completeness of reaction.

This background of information regarding the importance of the basic zinc-chromate complex crystal lattice structure, the apparent synergism of zinc and chromium, and the necessity of completeness of reaction provided the basis for further field tests.

TABLE IX

POTATO YIELDS IN BUSHELS PER ACRE, 1946

Fungicide	Area and potato variety			
	Concn., lb./100 gal.	Pennsylvania, Russet Rural	West Virginia,* Irish Cobbler	Florida,** Katahdin
Check	—	138	122	79
Bordeaux	8–8	227	179	—
Dithane D-14†	2 qts.	202	178	103
Tribasic‡	4	219	227	96
Cu-Zn-Cr (169)	4 to 6	202	232	110

* Data of Dr. J. G. Leach.
** Data of Dr. G. R. Townsend.
† Disodium ethylenebisdithiocarbamate $+ ZnSO_4 + Ca(OH)_2$.
‡ Tribasic copper sulfate.

In 1946, confirmatory tests in West Virginia with Dr. J. G. Leach (9), and in three areas in Ohio with Dr. J. D. Wilson, in addition to the State College, Pennsylvania, tests showed that copper-zinc-chromate complex, coded 169, at 4 to 6 lb. plus DDT at 3/4 lb. active per 100 gallons was consistently among the top three high-yielding treatments regardless of whether Early Blight, Late Blight, or both diseases were yield-controlling, or whether there were no observable fungus diseases. No other fungicide tested was so consistent over such a wide geographical range or range of disease conditions. Potato yields for these areas are given in Tables IX and X.

It was apparent that zinc was necessary but not in as high concentration as when a high degree of insect repellency was sought, *i.e.*, before the ad-

TABLE X

POTATO YIELDS IN BUSHELS PER ACRE, OHIO, 1946*

Fungicide	Concn., lb./100 gal.	Wooster	Marietta	McGuffey
Check	—	220	219	398
Bordeaux	8–4	365	491	548
Zerlate**	2	414	498	540
COCS†	4	356	481	563
Cu-Zn-Cr (169)	4 to 6	381	493	583

* Data of Dr. J. D. Wilson.
** Zinc dimethyldithiocarbamate.
† Copper oxychloride sulfate.

vent of DDT. It appeared advisable to have as high a copper content as could be obtained while still retaining the basic zinc-chromate complex crystal lattice structure essentially completely reacted. On this basis, a medium high-copper low-zinc-chromate complex was prepared and coded 658.

Field tests in 1947 in widely separated areas (8) and under a wide variety of disease conditions established that 2 to 3 lb. of 658 was equivalent to 4 to 6 lb. of 169. This is shown in Tables XI and XII.

TABLE XI

POTATO YIELDS IN BUSHELS PER ACRE, 1947

Fungicide	Concn., lb./100 gal.	Area and potato variety			
		Pennsylvania		West Virginia*	
		Russet Rural	Katahdin	Irish Cobbler	Sebago
Check	—	243	318	312	320
Bordeaux	8–4 or 8–8	315	363	293	300
Dithane D-14**	2 qts.	341	377	265	326
Parzate†	2	339	374	—	—
Tribasic††	4	328	382	297	242
Cu-Zn-Cr (658)	2 to 3	320	390	324	307
Cu-Zn-Cr (169)	5	288	359	286	—

* Data of Dr. J. G. Leach.
** Disodium ethylenebisdithiocarbamate $+ZnSO_4+Ca(OH)_2$.
† Zinc ethylenebisdithiocarbamate.
†† Tribasic copper sulfate.

The copper-zinc-chromate complexes are not simply copper fungicides as is indicated in Table XIII where yield is compared with total copper content. Potatoes sprayed with chromates containing little over one-third the total copper content of so-called fixed coppers or Bordeaux mixture yielded as much as these standard treatments. Continued efforts are being made to improve further the copper-zinc-chromate complexes by addi-

TABLE XII

POTATO YIELDS IN BUSHELS PER ACRE, OHIO, 1947*

Fungicide	Concn., lb./100 gal.	Areas		
		Wooster	Marietta	McGuffey
Check	—	354	365	644
Bordeaux	8–4	445	491	815
Dithane D-14**	2 qts.	473	540	854
Parzate†	2	453	502	856
Tribasic††	4	494	496	789
Parzate alternating with Zerlate‡	2	550	566	844
Cu-Zn-Cr (658)	2 to 3	518	586	806

* Data of Dr. J. D. Wilson.
** Disodium ethylenebisdithiocarbamate+$ZnSO_4$+$Ca(OH)_2$.
† Zinc ethylenebisdithiocarbamate.
†† Tribasic copper sulfate.
‡ Zinc dimethyldithiocarbamate.

TABLE XIII

RELATION OF COPPER CONTENT TO POTATO YIELDS

Fungicide	Concn., lb./100 gal.	Copper, lb./100 gal.	Yields in bushels per acre—1947		
			Pennsylvania		West Virginia*
			Russet Rural	Katahdin	Irish Cobbler
Tribasic**	4	2.12	328	382	297
Bordeaux	8–8	2.03	315	363	293
Cu-Zn-Cr (169)	3	0.30	288	359	286
Cu-Zn-Cr (169)	5	0.50	—	—	314
Cu-Zn-Cr (658)	2	0.60	307	366	319
Cu-Zn-Cr (658)	3	0.88	329	382	307

* Data of Dr. J. G. Leach.
** Tribasic copper sulfate.

tional changes in the ratio and the nature of the components. It is to be hoped that even more efficient fungicides will be developed in the future.

SUMMARY

Laboratory and greenhouse bioassays indicated the fungitoxic properties of the chromate complexes and established the range of chromate solubility useful on plants in foliage to be below 0.03 per cent to avoid phytotoxicity and above 0.0001 per cent to insure fungitoxicity.

When suspended in water, the solubilities of the component metals in the chromate complexes increased with the concentration of the suspension and their molar ratios differed in solution from their original molar ratios in the solid. This indicated that the complexes did not go into solution as units but that water acted to change their compositions.

This was reversible; when a suspension was concentrated by vacuum evaporation, the dissolved part of the complex reformed the solid phase again with the same solubility characteristics. These properties indicated that the basic chromates were non-Daltonian in character, *i.e.*, distinct and reproducible chemical entities the ratios of whose components are capable of being varied continuously over a wide range of composition.

Although a copper-chromate complex controlled fungus diseases of potato in the field, the plots failed to yield well. Zinc-chromate complex controlled tip burn of potatoes by repelling leafhoppers. Copper-zinc-chromate complexes controlled tip burn more efficiently and therefore yielded better than did zinc-chromate complexes. Tank mixtures of copper-chromate complex and zinc-chromate complex performed as a straight zinc-chromate complex.

Low-copper high-zinc-chromate complex, coded 169, used at 4 to 6 lb./100 gal. controlled fungus diseases and yielded as well as Bordeaux mixture.

With the advent of DDT as a potato insecticide, insect repellent properties were no longer necessary. Copper-chromate complex plus DDT did not yield as well as copper-zinc-chromate complex plus DDT confirming the desirability of zinc for reasons other than insect repellency.

X-ray diffraction studies of chromate complexes indicated a pattern common to all the copper-zinc-chromates identifiable with that of basic zinc-chromate. They differed only in sharpness of lines and shifting of interplanar distances. This indicated contraction of the crystal lattice as more and more zinc was replaced by copper, and was true for all proportions of zinc and copper up to 0.8 zinc to 4.2 copper to 1.375 chromium. Beyond this, the basic zinc pattern was unchanged but new lines were superimposed.

Performance as potato fungicides was found to be correlated with ratios of zinc to copper to chromium, and completeness of reaction of the product. A medium high-copper low-zinc-chromate complex, coded 658, was as efficient at about 2 lb./100 gal. as 4 to 6 lb. of low-copper high-zinc-chromate complex, coded 169. Chromates at little more than one-third the copper content of fixed coppers and Bordeaux mixture were shown to be more effective over a wide variety of geographical and disease ranges.

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THE ROLE OF THE SURFACE MICRO-FLORA IN MEASUREMENTS OF THE RESPIRATION RATE OF GERMINATING SEEDS

F. E. Denny

When plant organs are placed in a container for a respiration measurement, the total CO_2 produced will of course include that due to any microorganisms on or in the tissue. Is it likely that this micro-flora contributes an important share of this total?

Dolk and van Slogteren (2) found that measurements of the respiration of diseased hyacinth bulbs were complicated by the presence, on the surface of the bulbs, of bacteria, the respiration of which was greater than that of the bulbs themselves. They believe, furthermore, that the increase in respiration undergone by tissues that receive the "warm bath" treatment for breaking bud dormancy is due not to an effect upon the host plant but rather to one upon the growth of the surface microorganisms. They regard it as imperative to measure respiration under sterile conditions.

Much work has been done in this field by investigators of factors in the storage of grains. It appears that if the humidity in the bin is high enough to favor growth of microorganisms the respiration found is due mainly to them. Thus, Leach (4) reports: "Where unlimited absorption of water and germination of wheat are prevented, the carbon dioxide production by the grain is due almost entirely to the respiration of the microorganisms that infect it," and according to Milner (5) "the carbon dioxide evolved from normal grain, held over time intervals of several days at moisture levels in hygroscopic equilibrium with air relative humidities in excess of 75 per cent, is due principally to the respiration of an indigenous mold population growing on the seeds." In the storage of soybeans, Milner and Geddes (6, p. 239) found that "when the moisture content exceeds a minimum value (usually about 14% but dependent on a number of factors), a very sharp increase in the respiratory rate of soybeans occurs; this indicates that an additional and very active agency contributing to the respiration has come into play. The evidence is quite conclusive that molds are responsible."

In the experiments just described the moisture content of the seeds was not high enough to initiate active growth of the seedlings. The conditions were unfavorable for increasing the respiration rate of the seeds themselves, but were favorable for the development of the micro-flora. In many cases, however, the main interest would be in the respiration of seeds

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during a period of active growth of the young seedling, i.e. during the early stages of germination.

Nabokich (7) dealt with this phase of the problem. He estimated that sterile bean seeds artificially infested with organisms from the soak-water of seeds respired about 16 to 25 per cent faster than a comparable lot of sterile seeds. Fernandes (3), however, working with pea seeds came to the conclusion that the apparent decrease in the respiration of the seeds treated with the sterilizing agent (mercuric chloride in this case) was due to the injurious effect of the agent on the pea seeds themselves, and that the microorganisms were contributing negligibly to the total CO_2 production.

The present experiments were restricted to seeds, since any samples taken would almost certainly be well-stocked with organisms. They were also restricted to the early stage of germination which occurs when fully imbibed seeds are placed under germinating conditions at about 20° C., since the action of factors with respect to the dry seeds had already been well established by the cited workers in the field of grain storage.

It was felt that it was unnecessary to obtain complete sterility of the seed surfaces, but that if a treatment could be found that would merely retard the development of microorganisms for one day, or at most two days, the practical requirements of the experiment would be met. In other words, "disinfestation" rather than "disinfection" was sought for, and by "disinfestation" is meant (at least in this paper) any action which prevents the multiplication of microorganisms, whether by a "toxic" or a "static" action, and whether the organisms are fungi, bacteria, or yeasts.

It seemed particularly desirable to avoid the use of such agents as mercuric chloride or silver nitrate, because of their toxic action, but to test some of the newer synthetic fungicides which have high fungicidal or fungistatic activity with little injurious action upon the host plant.

It appeared that a most essential requirement for any disinfestant to be considered suitable for consideration would be not only that it must have no injurious action on the young seedling, but more particularly it should have no effect upon the rate of carbon dioxide production of the treated seedling apart from any possible effect on the micro-flora.

It is believed that chemical treatments fulfilling these requirements were found, and that the tests were replicated enough times to furnish a reasonably good error term for determining the significance of any differences found.

Briefly stated, the results obtained indicate that in some cases a distinctly observable excess respiration rate due to the presence of contaminating organisms could be shown to occur, while in other cases such increased rates were not found. The deliberate addition to the seed surfaces of a liquid known to contain a large number of microorganisms in nearly every case resulted in an increased production of carbon dioxide. While these changes occurred, the relative effect upon the values for CO_2 produced were not large, usually not more than about 10 per cent either below or above the rate of the control lots.

MATERIALS AND METHODS

Seeds. The seeds used were: wheat, *Triticum aestivum* L., Leap's Prolific; buckwheat, *Fagopyrum esculentum* Gaertn.; bush bean, *Phaseolus vulgaris* L., Tendergreen; barley, *Hordeum vulgare* L., Alpha; rye, *Secale cereale* L.; field corn, *Zea mays* L., Early Golden Orange Dent. All of these lots of seeds were of good quality and gave high percentages of germination and emergence, except the rye, which was an old sample, very dusty, and showed only about 16 per cent emergence.

The seeds were weighed out in small samples, each tied in a cheesecloth bag, using a bag sufficiently large to permit maximum swelling in water, and yet allow at the end of the soaking period plenty of space for the seeds to be brought in contact with the liquids into which they were to be dipped. Seeds were soaked in water for 20 to 24 hours at 20° C. before being placed in the respiration chambers.

After the bags of seeds were dipped into the chemical solution or suspension, or in other liquids, they were placed on blotting paper to drain, the draining period being usually about one hour.

The seeds were in the early stages of germination in the succeeding period in the respirometers, approximately 24 hours except as otherwise stated in the text.

Chemicals. A preliminary test was made of many chemicals, but finally the list was narrowed down to four. Three were chemicals recently developed especially for use with seeds against fungi causing disease or damping-off, these being effective at low concentrations and also showing relatively low toxicity to the host plant. The three were: Spergon, effective constituent tetrachloroquinone manufactured by the United States Rubber Company, Naugatuck Chemical Division, New York, New York, the wettable form being used in these tests; Puratized N5E, effective constituent phenylmercuritriethanol ammonium lactate, manufactured by Puratized Inc., Windsor, Vermont, a subsidiary of Gallowhur Chemical Corporation; and Tersan, effective constituent tetramethylthiuram disulfide, manufactured by duPont Semesan Company, Inc., Wilmington, Delaware.

When these three chemicals were stirred with water in preparing a dipping liquid for seed treatments, Puratized formed a true solution, but Spergon and Tersan each gave a suspension. These liquids were stirred vigorously when the bags of seeds were placed in them so that a good film of chemical was left adhering to the seeds and to the cloth bag. This film was left on during the course of the test and it was depended upon to take care of the air-borne organisms, so that the test could be carried on without using sterile containers and without provisions for the maintenance of a sterile environment.

The fourth chemical selected was calcium hypochlorite, $Ca(OCl)_2$, and this was done on account of the general use which has been made in plant physiological studies of this chemical for sterilizing seeds or other tissues. Wilson (12), who first suggested its use, recommended a strength of $Ca(OCl)_2$ sufficient to furnish about 1 to 2 per cent available chlorine. Seeds were to be soaked for several hours in an excess of solution. Spaeth and Afanasiev (10) found for a number of seeds that this treatment delayed germination and reduced the percentage of germination. The attempt has been, heretofore, to produce complete sterilization so that the tissue could then be used in subsequent experiments. In such cases a delay in germination, or even the death of a few seeds or seedlings would have no serious consequences. In the present tests complete sterility was not sought for, nor was it necessary. The need was merely to retard the development of microorganisms for a day, or at most for two days, while a respiration test was carried out. It was found that for this purpose much lower amounts of calcium hypochlorite were effective, the required available chlorine concentration not being more than about 0.08 g. per 100 cc. instead of 1 or 2 g. as originally recommended by Wilson.

In these tests, the available chlorine content of the stock of calcium hypochlorite was determined according to the U. S. Pharmacopoeia (8, p. 503) and the amounts stated in the text and tables are grams of available chlorine in the amount of calcium hypochlorite taken. This solution was filtered through paper before being used.

Respiration apparatus. The method used in measuring the carbon dioxide output of the samples of soaked seeds is described in a previous article (1). See description for Method I in the cited article. Glass fruit jars, 2-quart size, were used as respirometers. The bags of seeds were placed on the screen supported by a shelf with glass-rod legs. For absorbing the CO_2 produced, a layer of 2 N NaOH solution (usually 50 cc.) is pipetted into the jar. Through the No. 13 rubber stopper of the jar a short capillary tube leads to a *t*-tube through which a slow current of oxygen is passing continuously. As fast as O_2 is consumed in the jar an equal quantity of O_2 is drawn into the jar from the *t*-tube. In this way the O_2 concentration in the jar is maintained at an approximate value of 20.9 per cent (with some variations as shown in the cited article). The jars are placed in position on a wooden rack of a post office box type, the *t*-tubes from the jars being connected in series, the O_2 supply then being available to each of the jars in the test. The rack accommodated 36 jars at a time. At the end of an experimental period, the alkali was rinsed into a 500 cc. volumetric flask, a quantity of saturated barium chloride solution sufficient to cause complete precipitation was added, and after filling to the mark, mixing, and allowing the precipitate to settle, aliquots were taken for titration against either 0.5 or 0.25 N HCl.

Temperature. The respiration measurements were made in a constant temperature room at 20° C., maintained continuously by thermostatic control.

SELECTION OF CHEMICAL TREATMENTS FOR DISINFESTING SEEDS

CONCENTRATION OF CHEMICALS REQUIRED TO INHIBIT GROWTH OF ORGANISMS

The following method was used in arriving at an estimate of the effectiveness of various chemicals in inhibiting growth of such organisms as happened to occur on the seeds. To a nutrient agar containing the desired amount of chemical were added a few cc. of a liquid obtained by soaking seeds in a small quantity of water for a day or more at room temperature. The added liquid contained large numbers of organisms that had developed on the seed surfaces and in the soak-liquid, and these were distributed throughout the agar medium along with the added chemical at the time the plate was poured. The Petri dishes were then stored at 20° C., and were examined at intervals thereafter.

It should be emphasized that no preliminary sterilization of the Petri dishes or of the culture medium was carried out, nor was any attempt made to prevent contamination from the air. The agar medium was not autoclaved, but was merely heated in a boiling-water bath in the open air; it was removed from the bath as soon as the medium became melted and in condition to pour; after the plate was poured, the cover was put on to prevent drying out but the pouring of the plate took place in the open air of the laboratory with good opportunity for inoculation from the air. The only factor bringing about sterility or inhibition of growth of the microorganisms present was that furnished by the chemical itself.

The results of such a test are shown in Figure 1. A Spergon suspension of either 0.6 or 0.3 g. per 100 cc. (Fig. 1 A I, II) prevented the growth of organisms on the plate over a period of three days following inoculation, and 0.3 g. remained satisfactory for five days (Fig. 1 A, III); indeed, even when the plates were held for 10 days after inoculation no spots appeared (not shown in Fig. 1) on the plates with 0.3 g. of Spergon per 100 cc. The milky background on the Spergon plates is not caused by growth of organisms, but by particles of Spergon suspended in the medium.

Calcium hypochlorite, $Ca(OCl)_2$, at concentrations of either 0.16 or 0.08 g. of available chlorine per 100 cc. of culture (Fig. 1 B) prevented growth over a three-day period, but at the end of a five-day period (Fig. 1 B III) scattered growth began, and by the seventh day growth started

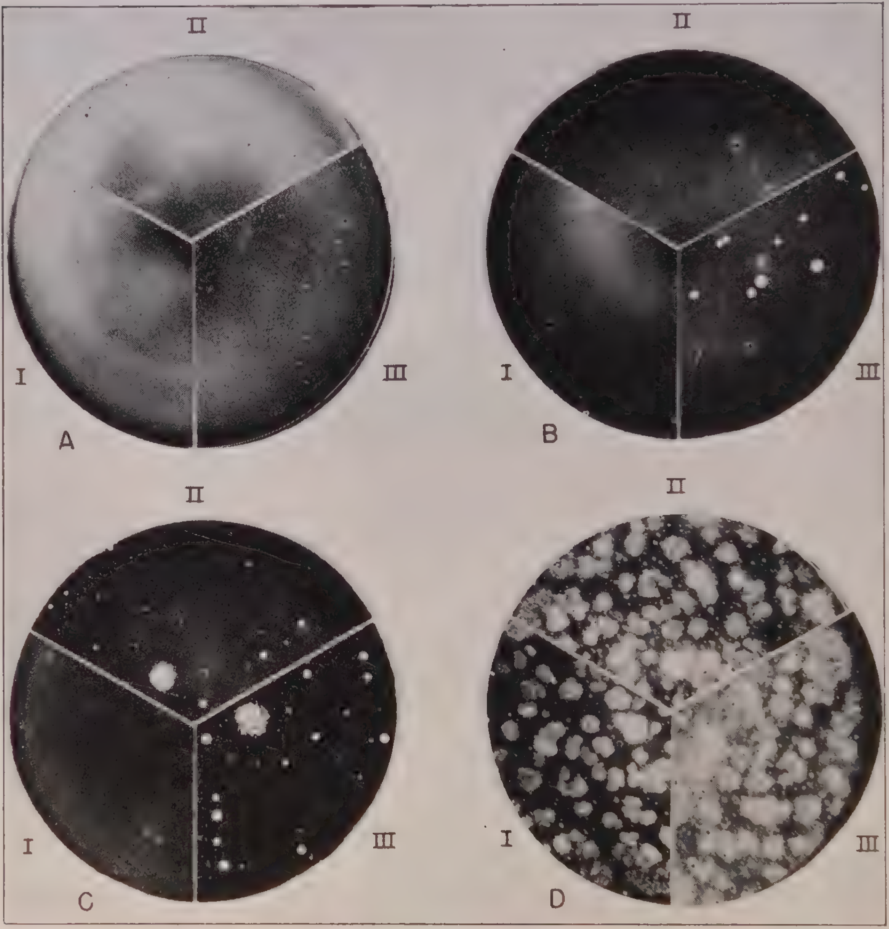

FIGURE 1. Photographs of agar plates (one-third of a print) showing the effectiveness of chemicals in preventing growth of microorganisms. Medium consisted of 2 g. of agar, 2 g. of dextrose, 50 cc. of potato extract, 35 cc. of water containing the indicated amount of chemical, and 15 cc. of a "soak-liquid" obtained by soaking 100 g. of wheat seeds for one day in 150 cc. of H_2O, then decanting and rinsing, to a final volume of 150 cc. A—Spergon; I, 0.6 g. per 100 cc. of medium, 3 days after pouring; II, 0.3 g. after 3 days; III, same as II but photographed 2 days later than II. B—Calcium hypochlorite; I, 0.16 g. of available chlorine per 100 cc., 3 days after pouring; II, 0.08 g. after 3 days; III, same as II but photographed 2 days later than II. C—Puratized; I, 0.05 g. per 100 cc., 3 days after pouring; II, 0.025 g., 3 days after pouring; III, same as II but photographed 2 days later than II. D—Control (35 cc. of water without chemical added, but the 15 cc. of soak-liquid was included); I, 3 days after pouring; II, same as I but photographed 2 days later than II; III, same but photographed 4 days later than II. All Petri dishes stored at 20° C. continuously.

even on the plate (not shown in Fig. 1) with the higher amount of chemical.

With Puratized (Fig. 1 C) no growth occurred with 0.05 g. per 100 cc. of nutrient agar, but with 0.025 g. there was contamination of the plate after three days, and with some increase in the number of spots after five days; a photograph taken at the end of seven days (not shown in Fig. 1) indicated no further increase in the number of colonies, and very little increase in the diameter of those already formed. Although some growth of organisms occurred in the lowest concentration of Puratized, comparison with the control lot shows that multiplication of organisms was effectively checked, and that the growth of fungi (perhaps the form which is particularly active under these conditions in producing CO_2) was completely inhibited.

Results with Tersan (not shown here) using 0.3 and 0.15 g. per 100 cc. were similar to those with Spergon.

The control plates (Fig. 1 D) show that large numbers of organisms were present in the agar, and that the conditions in the Petri dishes were favorable for continued growth at least up to seven days from the time of inoculation.

Figure 1 demonstrates the effectiveness of these chemicals in inhibiting completely, or at least in retarding strongly, the growth not only of the organisms present on the seeds, but also of those occurring on the non-sterile glassware, and of those falling in from the air.

Spergon and calcium hypochlorite were chosen for tests to determine the lower limits of concentrations of chemical. The inoculating liquid was obtained by combining the soak-liquids from seeds of both wheat and rye. These results are shown in Figure 2. When the amount of Spergon used was reduced from 0.15 g. per 100 cc. to 0.075 g. a single spot was found on the Petri dish after two days (Fig. 2 B), and with a further reduction to 0.0375 g. several spots occurred (Fig. 2 C), but even at this low concentration good control was obtained, especially with regard to the multiplication of fungi (compare Fig. 2 C with the control Fig. 2 G). After reducing the concentration of calcium hypochlorite from 0.08 g. available chlorine per 100 cc. to half strength, control of organisms was still obtained (Fig. 2 E), but upon lowering the concentration to 0.02 g. available chlorine per 100 cc. growth of both fungi and non-fungi occurred (Fig. 2 F).

EFFECT ON SEED RESPIRATION OF A RANGE OF CHEMICAL CONCENTRATIONS

It is necessary to know whether the chemical treatments of the seeds are exerting a direct effect on the respiration, apart from any effect on the growth of organisms, i.e. whether the treatments are themselves retarding the respiration of the seeds. It was decided that one way of obtaining information on this point would be to treat seeds with different concentrations and note what the relation is between concentration of chemical and respiration rate. If the respiration is about the same over a rather wide concentration range, this fact is evidence against the view that the treatment is retarding the respiration.

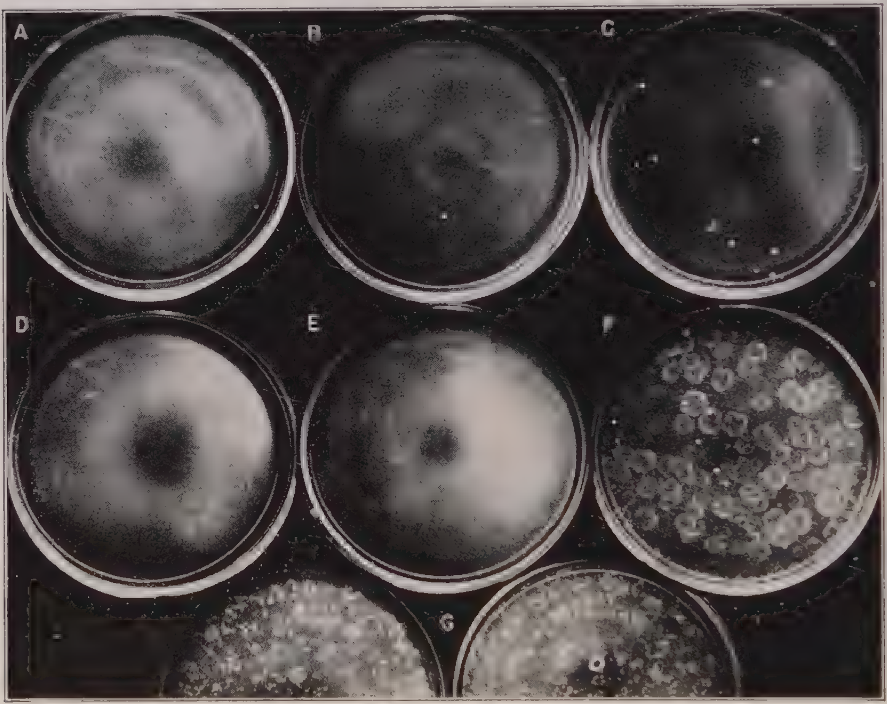

FIGURE 2. Effect of very low concentrations of Spergon and calcium hypochlorite on the growth of microorganisms. Preparation of agar medium same as for Figure 1. The 15 cc. of soak-liquid added was obtained by soaking wheat seeds and rye seeds in H_2O for one day, decanting and mixing the soak-liquids from the two sources. A, B, C—Spergon; A, 0.15 g. per 100 cc. of medium; B, 0.075 g.; C, 0.0375 g. D, E, F—Calcium hypochlorite; D, 0.08 g. of available chlorine per 100 cc. of medium; E, 0.04 g.; F, 0.02 g.; G—Control, receiving no chemical but with the 15 cc. of soak-liquid added, print shown cut in two to conserve space. All photographed 2 days after pouring.

Three concentrations of each of three chemicals were chosen for this test. The lowest concentration in each case was that indicated in the preceding section (see also Fig. 1) as being well toward the lower margin for effectiveness in inhibiting growth of microorganisms. The two higher concentrations were twice and four times this. The results of such a test with six different kinds of seeds are given in Table I. With all seeds tested there were no differences among the three concentrations.

With all but rye seeds, there were no differences among the three chemicals. This fact, in itself, lends support to the view that the chemical treatments are not exerting a direct retarding effect upon the respiration of the seeds. If the chemicals were affecting the respiration, it is unlikely that the three would behave so similarly with five out of six of the seeds tested.

The result with calcium hypochlorite and rye is anomalous. This sample of rye was an old one, the seeds germinating to the extent of only about 16 per cent, and it may be that the micro-floral population contained species against which $Ca(OCl)_2$ was effective while the other two chemicals were not. But although $Ca(OCl)_2$ differed from the other chemicals with respect to rye, it lined up satisfactorily for the other five species, and there is no reason to suspect it in those cases.

TABLE I

EFFECT UPON RESPIRATION OBTAINED BY DIPPING SOAKED SEEDS IN DIFFERENT CONCENTRATIONS OF DISINFESTING CHEMICALS

Chemical	Concn., g. per 100 cc.	Respiration, cc. CO_2 (at 0° C., 760 mm.) produced in 23–25 hours					
		Wheat, 56 g.	Buck-wheat, 56 g.**	Bush bean, 60 g.	Rye, 50 g.	Barley, 50 g.	Field corn, 100 g.
Spergon	1.2	167	78	245	157	185	154
	0.6	174	80	254	165	191	151
	0.3	171	88	251	165	188	142
Puratized N5E	0.1	157	83	257	133	165	142
	0.05	151	83	248	142	165	142
	0.025	170	78	248	155	171	137
Calcium hypochlorite	0.32*	161	66	239	91	171	148
	0.16*	152	68	257	101	177	137
	0.08*	151	78	262	91	168	142

* G. of available chlorine per 100 cc.

** This seed was from another source and had a distinctly lower respiration rate than the seed in Table II.

The conclusion from Table I is that it is unlikely that the chemicals are causing a retardation of the respiration of the seeds treated with them.

EFFECT OF CHEMICAL TREATMENTS ON GROWTH OF SEEDLINGS

After the soaked seeds had been treated with the chemicals, and after the respiration measurements over a 24-hour period had been completed, the seedlings were removed from the respirometers, were rinsed in running water, and were planted in soil in flats. This was to observe the early growth of the seedlings after the chemical treatment, to note whether there was

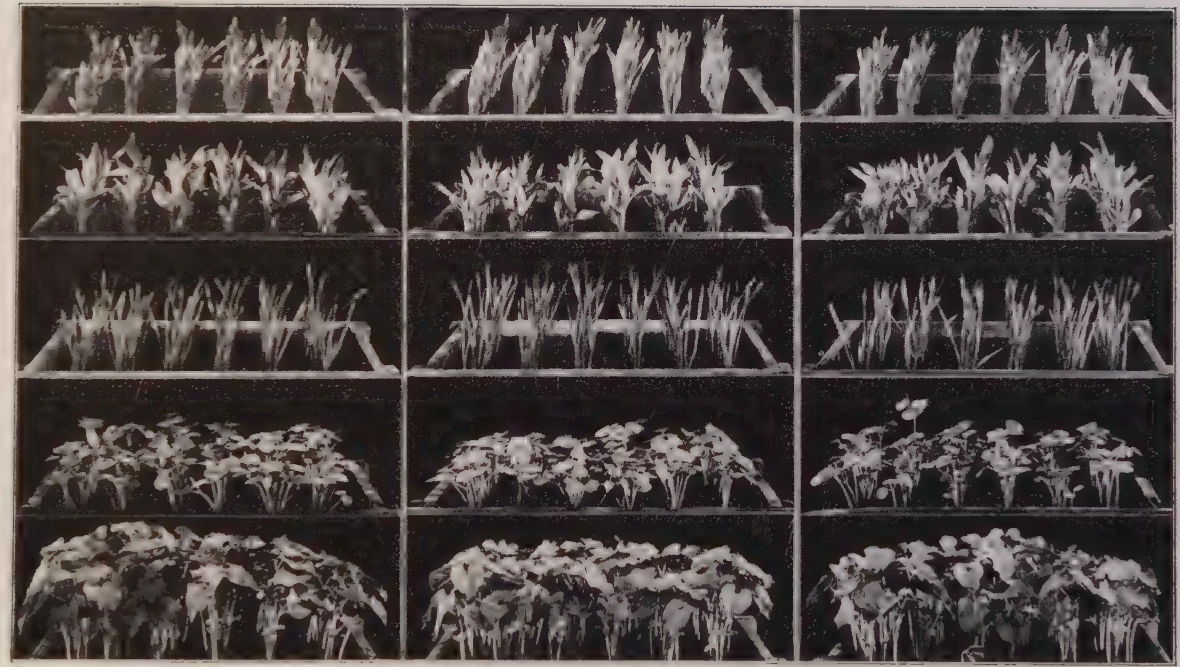

FIGURE 3. Subsequent early growth of seeds treated with chemicals and held in respirometers for one day, after which the seeds were rinsed in running tap water and planted in soil in flats. Row 1—barley; row 2—field corn; row 3—wheat; row 4—buckwheat; row 5—bush bean. Column 1—Spergon; column 2—calcium hypochlorite; column 3—Puratized. In each flat left to right, first 2 rows highest concentration of chemical, second 2 rows medium concentration of chemical, third 2 rows, lowest concentration of chemical. The concentrations of chemicals used were: Spergon, 1.2, 0.6, 0.3 g. per 100 cc. of dipping liquid; calcium hypochlorite, 0.32 g., 0.16 g., 0.08 g. of available chlorine per 100 cc.; Puratized, 0.1, 0.05, 0.025 g. per 100 cc.

any evidence of toxicity of the chemical used for disinfesting, or any differences between the concentrations tested.

The results are shown in Figure 3. Five different seeds, three chemicals, and three concentrations of each chemical were involved in the test. No important differences were found among the chemicals and the concentrations. The seedlings appeared to be normal and to grow thriftily. The results are in favor of the view that the chemical treatments used for disinfesting the seeds had no distinctly injurious effect upon the growth of the seedlings, at least in the period shortly after the treatment.

RESPIRATION RATES OF INFESTED AND DISINFESTED SOAKED SEEDS

EXPERIMENTS WITH WHEAT AND BUCKWHEAT

The previous paragraphs have shown that the selected chemicals were effective in inhibiting, completely or at least largely, the growth of organisms; that the treatments were not injurious to the seeds as shown by the subsequent growth of the seedlings (at least in the early stages); and that the chemicals were not themselves exerting a retarding effect on respiration.

Although there was a considerable range of concentrations within which the preceding statements held good, it was decided to work with the lowest possible concentration of each chemical, in order to avoid the principal criticism previously made of experiments of this type, namely that the lowered respiration resulting from disinfesting the seeds was due to an injurious action of the chemical on the treated seeds.

The chemicals and concentrations chosen for these tests were: Spergon, 0.3 g. per 100 cc. of water; Puratized N5E, 0.025 g. per 100 cc.; Tersan, 0.15 g. per 100 cc.; calcium hypochlorite, $Ca(OCl)_2$, 0.08 g. of available chlorine per 100 cc. Calcium hypochlorite (after filtration) and Puratized gave clear solutions, but Spergon and Tersan were used as suspensions. The seeds to be treated were tied in over-sized cheesecloth bags in order that after the preliminary soaking period the seeds would not be squeezed in a tight ball but would be loose in the bag to allow good distribution of the chemical solution or suspension. Care was taken to stir constantly while the seed sample was being dipped. After dipping, the bags of seeds were placed on blotters for about an hour to drain thoroughly.

The results of such a test for wheat and buckwheat are shown in Table II. There were quadruplicate samples for each treatment in each series. Column 2 shows that there were seeds from two different sources of each type, and it should be emphasized that these were distinct lots of seeds from different sources, and not merely two different samples from the same stock of seeds. The soak-liquid referred to in columns 7 and 8 was obtained as follows: To each 100 g. of dry seeds were added 45 cc. of H_2O; this was

TABLE II

EFFECT ON RESPIRATION OBTAINED BY DIPPING SOAKED SEEDS IN INFESTING AND DISINFESTING LIQUIDS

Seed, and weight in g.	Seed from source, No.	Respiration rate, cc. of CO_2 (at 0°, 760) produced in 23 hrs. by dipping soaked seeds into the following liquids						
		Spergon	Puratized	Tersan	$Ca(OCl)_2$	Soak liquid from		Water
						Wheat	Buckwheat	
Wheat, 64 g.	I	172	169	173	185	217		202
		181	179	168	194	224		196
		194	175	173	194	227		205
		186	179	182	189	233		205
		733	702	696	762	901		808
	II	187	178	184	168	211		200
		187	181	187	168	212		204
		189	174	185	174	211		201
		191	184	187	181	222		211
		754	717	743	691	856		816
Buckwheat, 64 g.	I	197	197	200	181	202	201	205
		200	195	200	185	195	194	204
		192	187	200	189	205	194	194
		192	182	185	189	202	188	197
		781	761	785	744	804	777	800
	II	205	192	200	188	207	204	208
		198	187	191	177	195	198	195
		191	211	195	194	207	204	194
		200	198	198	191	218	201	189
		794	788	784	750	827	807	786

Mean squares and *F* values obtained in the analysis of variance

	Separate seed sources						Each pair of seed sources				
	Wheat			Buckwheat				D.F.	Wheat	D.F.	Buck.
	D.F.	I	II	D.F.	I	II					
D.F. and M.S. for Treat.	5	1504	962	6	111	138	Treat.	5	2219	6	217
" " " " Repl.	18	37	19	21	30	53	*T*×*S*	5	247	6	32
							Repl.	36	28	42	43
Over-all *F* values	—	41**	51**	—	3.7*	2.6*	—	—	9.0*	—	6.8*

F values for certain comparisons

Chem. vs. H_2O	1	39**	84**	1	7.0*	0.2	—	1	12.3*	1	4.8
Added organisms vs. H_2O	1	29**	10**	1	0.03	3.0	—	1	4.5	1	3.9
Disinfested vs. not	1	620**	208**	1	9.5**	6.2*	—	1	39**	1	19**

* Significant at the 0.05 level of probability.
** Significant at the 0.01 level.

allowed to stand at room temperature for about 65 hours; then the liquid was decanted, the seeds rinsed successively with small quantities of water, until the volume of liquid obtained by decanting and rinsing became 200 cc. Bags of seeds were dipped into this liquid and allowed to drain. The object was to increase the number of organisms on the seeds in the lots shown in columns 7 and 8. Not all of the increased number of organisms were actually on the seeds, some of the liquid being held in the cloth bags of these lots.

An examination of the data in Table II shows a tendency for the respiration of the lots treated with the chemicals to be low, and for the lots dipped in the soak liquids to be high. The control lots dipped into H_2O were intermediate, usually but not always greater than the treated and less than those with added organisms. These differences were definite and consistent with wheat but were less and rather uncertain with buckwheat˙

The values are sufficiently near to each other to require an analysis of variance. This can be done from two different points of view: (a) the results for each seed source may be analyzed separately, i.e. including all entries opposite wheat-I, wheat-II, buckwheat-I and buckwheat-II; (b) the two series for each species may be combined, i.e. including all entries opposite wheat and all opposite buckwheat.

The summary of some of the important values from the analysis of variance is given at the bottom of Table II. When the analysis related to the separate seed sources, the replicate variance was used as the error term for determining the *F* value, but for the analysis based on the combined data for the two seed sources the interaction variance, $T \times S$, was used. The over-all *F* values, i.e. 41, 51, etc. show significant differences for all comparisons made, although the value for buckwheat-II barely reaches the minimum requirement.

The last three lines in the analysis of variance are devoted to showing the *F* values for certain comparisons that are of special interest; i.e. whether there is a difference between the control dipped in H_2O and those dipped in the chemicals; whether adding organisms by dipping in the soak-liquid has increased the respiration over that shown by the control; and whether the disinfested lots (presumably with no contaminating micro-flora) have on the whole had a lower respiration than those with micro-organisms present, either as naturally-occurring or after being further contaminated by dipping in the soak liquid. The method used was that described by Snedecor (9, p. 402). For example, the comparison "chemicals vs. H_2O" for wheat-I, Table II, would be:

$$[(733+702+696+762)-(4)(808)]^2 \div (20 \times 4 \times 37) = 39.$$

Examining the *F* values in the line opposite the designation "chem. vs.

H_2O," it is seen that highly significant differences were found for wheat, but that with buckwheat only the entry for buckwheat-I reaches the required limit. The comparison "added organisms vs. H_2O" also showed high F values for wheat-I and wheat-II taken separately, the value for the combined pair, however, being only 4.5 and thus below the required value of 6.6. It may be that the error term, $T \times S = 247$, is unusually high. The last line in Table II shows the comparison "disinfested vs. not." The F values in this line are all above the minimum requirement for significance. As an example of the computation we have for buckwheat-II:

$$\frac{\left[[(3)(794)+(3)(788)+(3)(784)+(3)(750)] - [(4)(827)+(4)(807)+(4)(786)]\right]^2}{(84)(4)(53)} = 6.2.$$

The value 84 is equal to $3^2+3^2+3^2+3^2+4^2+4^2+4^2$; 4 is the number of items in the body of the table added together to give the totals (i.e. 794, 788, etc.); 53 is the mean square for replicates in the experiment with buckwheat-II.

It is believed that Table II shows differences between the control lots and those receiving chemical treatments to inhibit the growth of microorganisms. These differences even when significant were, however, not large; with wheat, disinfesting the seeds reduced the respiration rate by about 11 per cent; with buckwheat-I the reduction in rate was only about 4 per cent, while for buckwheat-II no difference was found.

Also, while dipping the seeds into the soak-liquid in order to increase the number of contaminating organisms tended to increase the rate of respiration, these differences when found were not large. Thus, for wheat the increase was about 5 to 10 per cent, while for buckwheat no certain effect was obtained by increasing the number of organisms with soak-liquid from either wheat or buckwheat.

EXPERIMENTS WITH OTHER SEEDS

Results obtained with four other seeds are shown in Table III. In this experiment, since the previous tests indicated very little or perhaps no difference among the chemicals, only one disinfestant, Spergon, was used; again, a comparison was made with seeds to which added organisms were applied by means of a soak-liquid. Columns 5, 6, and 7 show the portion of the analysis of variance needed to obtain an estimate of the significance of the results. The F values in column 7 are all higher than 6.36, the minimum needed for significance at the 0.01 level of probability. In deciding which pairs of comparisons gave significant differences, the ranking method proposed by Wilcoxon (11) was found convenient. With each seed tested there was a significant increase in respiration resulting from dipping in the soak-liquid, this increase varying from about 8 per cent for beans and barley to about 20 per cent for rye. For the comparison between Spergon and the water control, however, a significant difference was found

TABLE III

EFFECT OF A DISINFESTANT AND A CONTAMINANT ON THE RESPIRATION OF SOAKED SEEDS

Seed, and pre-soak weight, g.	Cc. CO_2 (at 0°, 760) produced in 23.6 hours when soaked seeds were dipped into:			Analysis of variance		
				Mean squares for		*F* values
	Spergon	Soak-liquid	Water	Treatments (2 D.F.)	Repl. (15 D.F.)	
Bush bean, 45 g.	188	195	174	345.5	42.06	8.2
	195	191	181			
	195	198	195			
	194	202	185			
	194	202	181			
	185	211	192			
	1151	1199	1108			
Barley, 60 g.	208	214	205	664.5	21.86	30.4
	195	220	207			
	194	221	202			
	202	221	197			
	192	215	200			
	197	215	197			
	1188	1306	1208			
Rye, 50 g.	160	234	174	4014.5	73.9	54.3
	167	222	187			
	165	217	181			
	161	224	185			
	171	204	181			
	181	204	178			
	1005	1305	1086			
Field corn, 100 g.	174	182	161	684.5	26.06	26.2
	170	192	174			
	165	187	172			
	165	181	168			
	160	188	163			
	165	180	161			
	999	1110	999			

only with the rye seeds, and even then the reduction in rate of respiration due to treatment with the disinfestant was only about 7 per cent.

The results in Table III, therefore, tend to corroborate those in Table II, in showing that while there is evidence that there may be some increase in carbon dioxide production due to the presence of surface microorganisms, the error due to ignoring the contributing action of the micro-flora usually would not be large. Under the conditions of such experiments as these, the total CO_2 produced was furnished mainly by the seeds themselves. Whether the amount of CO_2 contributed by the microorganisms would need to be taken into account would depend upon the particular purposes of the experiment. If an estimate of the amount of the micro-floral respiration appeared to be needed, the methods used in the present tests seem to be competent to furnish a dependable answer.

SUMMARY

The experiments relate to the question whether, in measurements of the respiration of soaked seeds in the early stages of germination, the CO_2 produced by the micro-flora on the surface of the seeds contributes a significantly large proportion, perhaps enough to vitiate the results obtained in using non-sterile seeds.

The seeds used were wheat, buckwheat, rye, barley, field corn, and bush bean, and all were pre-soaked 20 to 24 hours in water at 20° C. before being placed in the respirometers. Carbon dioxide production was measured (usually over a period of 24 hours) at 20° C.

The treatments were made by dipping the seeds in solutions or suspensions of chemicals, the film of liquid so deposited being allowed to remain throughout the test. Not total disinfection but merely a temporary disinfestation (i.e. inhibition of growth of microorganisms during a 24 to 48 hour period) was sought.

A chemical preparation was deemed satisfactory if it would prevent for at least two days visible growth of organisms in non-sterile Petri dishes, containing a non-sterile nutrient agar, into which was added just before solidification a liquid containing large numbers of organisms resulting from soaking seeds in water at room temperature for two to three days.

The following four chemicals (at suitable concentrations) were used satisfactorily: Spergon, Puratized N5E, Tersan, and calcium hypochlorite $Ca(OCl)_2$. Furthermore, tests showed that these chemicals at the concentrations used (or even at doubled or quadrupled values) had no direct effect upon the respiration rate (except possibly for $Ca(OCl)_2$ with rye), and had no injurious effect upon the subsequent early growth of the seedlings.

Significant differences between disinfested seeds and the controls (dipped into water instead of chemical) were found only with wheat and rye, and in one out of two tests with buckwheat. These differences, even with these seeds, were small, the CO_2 production of the control being only about 10 per cent higher than that of the disinfested lots.

In a concurrent series of tests, comparisons were made between the control lots dipped into water, and other lots dipped into a liquid ob-

tained by soaking seeds for 65 hours in water and decanting. This soak-liquid served to increase the number of microorganisms on the seed surfaces. With all seeds except buckwheat, the CO_2 production of the lots with added organisms was significantly higher than that of the control, the increases being, however, only about 10 per cent.

With these seeds under these conditions, therefore, the effect on CO_2 production due to the contamination with surface microorganisms has not been large, and vitiation of results from this source seems not likely. If small differences in an experiment become an important factor, the methods used seem capable of detecting and measuring them.

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RELIABILITY OF THE EXCISED EMBRYO METHOD AS A RAPID TEST FOR DETERMINING THE GERMINATIVE CAPACITY OF DORMANT SEEDS

Florence Flemion

Various methods have been suggested for the accurate estimation of seed viability. The usual method has been germination but with many seeds special treatment and long periods of time are required for results. A simple reliable method that will give the true planting value of seeds more quickly than germination tests is needed—one that will indicate germination energy as well as germination capacity.

A method has been described (10, 11) which is especially time-saving when testing seeds which require many months for germination to occur. Results obtained by observing the behavior of the excised embryos placed on moist filter paper for several days agree closely with actual germination tests. The percentage viable as well as some indication of vigor is obtainable within three to ten days for very dormant seeds and sooner for quick-germinating seeds. Any difficulty that may arise with this test is in the removal of the embryos which requires considerable care. However, various techniques (11, 64) have been developed which facilitate this process. The method has been tested on many kinds of seeds (23) and, including the data on 58 species representing 19 families presented in this study, various members of 31 plant families have thus far been studied. In all these cases the results have shown that the observation of the behavior of the excised embryo serves as a good test for the viability of the seeds.

MATERIALS AND METHODS

The seeds were collected in the vicinity of the Institute at Yonkers, New York, or obtained from either commercial seedsmen or private collectors.

The viability of the seeds was tested in two ways. Excised embryos were kept on moist filter paper in Petri dishes at room temperature (20° to 23° C.) and the behavior of these embryos was compared with the percentage germination obtained by following the recommended procedures.

Most of the embryos were white at the time of excision but there were a few exceptions as *Berberis* embryos were deep yellow, those of *Celastrus* were yellow-green, and *Orixa* were light green. Considerable variation as to size was encountered, as, for instance, the cotton embryos were about

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a half inch in length while embryos of *Lonicera Korolkovii* were so small that a low-powered binocular microscope was used to observe their behavior in the Petri dishes. Sometimes the cotyledons were coiled as with *Rhamnus* or wrinkled as with cotton. In some cases the endosperm was soft as in pines or extremely hard as in *Parthenocissus*.

In order to facilitate excising and to prevent injuring the embryo during the excision, the seeds should be fully imbibed with water. This was usually accomplished at room temperature by either soaking the seeds in tap water overnight or placing the seeds in small cheesecloth bags and keeping them in moist peat moss for several days. In cases where the seed coats were soft after soaking in water the embryos could be excised readily. Seeds of this type were those of *Arbutus*, *Caragana*, *Elaeagnus*, *Liquidambar*, *Salvia*, and *Silphium* as well as the permeable seeds of *Hibiscus*, okra, and *Robinia*. In the case of cotton seeds, a hole was pricked in the coat before the seeds were soaked. *Pyrus* seeds were very slippery when soaked but rubbing the seeds in cheesecloth removed this handicap.

Some embryos were more easily excised when, instead of soaking, the seeds were held in moist peat moss for several days. This method was used for many seeds including most of the members of the pine family. Also seeds of *Celastrus*, *Ligustrum*, *Lonicera dioica*, and *Syringa vulgaris* were held for three days in this condition while seeds of *Lonicera Korolkovii*, *Liriodendron*, *Orixa*, *Parthenocissus*, *Rhamnus*, and *Rosa rugosa* were held for seven days. Apparently during this period some changes occurred in the tiny embryos of *Lonicera Korolkovii* for they could then be much more easily differentiated from the surrounding endosperm tissue.

Even after a period of three or seven days in moist peat moss the coats may still be difficult to remove and additional treatment may be necessary. In order to remove the outer coats of *Parthenocissus* two slits were made in the coats over the embryo and when this cut section was pried off the exposed white embryo could be distinguished readily from the hard bluish-white endosperm. Clipping part of the coats off, so that the scalpel could act as a wedge to separate the two halves of the outer coats, was the method used for seeds of *Juniperus virginiana*, *Pinus caribaea*, *Pinus taeda*, and some lots of *Larix europaea*. With some lots of *Juniperus virginiana* an acid treatment prior to the period in moist peat moss was very helpful. *Taxodium* seeds varied a great deal but in most cases the embryos were easily excised after a period of seven to ten days at 5° C. (41° F.) in moist peat moss.

The hard outer coats of Mahaleb cherry and the *Cornus* spp. were removed with the aid of a seed-cracking machine (9, p. 290). When the intact seeds had imbibed water either by soaking in water or by being in moist peat moss for a few days, the embryos were no longer brittle and very little injury occurred when the hard coats were cracked. Also the embryos could be excised as soon as the outer coats were removed.

Seed samples sometimes contained mixtures of soft and hard seeds such as some lots of okra, *Hibiscus*, *Rhus*, and *Robinia*. After soaking the seeds in water for about 16 hours the soft seeds which had imbibed water and were swollen were excised. The hard seeds when thoroughly dried were either filed or treated with concentrated sulfuric acid. If, after subsequent soaking in water, hard seeds still remained, they were again treated with acid but this second treatment was usually for a much shorter period of time.

When excising embryos sometimes both coats were removed together. In other cases after the outer coats were removed slits were made with a sharp instrument in the inner coats and endosperm and usually the embryos could then be excised. But with *Celastrus* and *Rhamnus* after the slits were made the seeds were placed in water and after about an hour the embryos either floated or were easily removed.

As the embryos were removed they were placed in water and when all of a given lot had been excised they were transferred by means of a brush, scalpel, or forceps, depending upon the size of the embryo, to moist filter paper in a Petri dish (10 pieces of 15 cm. paper and 30 cc. of tap water) and kept at room temperature. During the summer months when the laboratory temperature was too high, the cultures were placed in a 20° C. controlled oven. Too much moisture is not conducive to good development of some of the embryos such as those of the Pinaceae and Vitaceae families and in such cases only 20 or 25 cc. of water was used.

By observing the behavior of the excised embryos an indication of viability could be obtained within 2 to 10 days. Dead embryos quickly deteriorated while viable embryos either developed or remained firm and intact. Those that remained firm usually retained their original color but occasionally some pigmentation developed. When the embryos exhibited development some indication of vigor could be observed by the rapidity and degree of development.

For the excision tests lots of 50 seeds each in duplicate and frequently in quadruplicate were used. Since embryos only were used in the Petri dishes, corrections were made when some of the seeds were empty or contained deteriorated or shriveled embryos. The percentage of non-viable seeds was readily determined by combining the per cent of worthless seeds encountered during excision with the per cent of embryos which deteriorated while on the moist filter paper.

For the germination tests duplicate lots of 100 seeds each were used. Seeds of some species germinated very quickly while others required several months of special treatment before germination could occur. When planted and kept in the greenhouse (70° F.) the seeds were sown in large pots containing a soil mixture of one-third sand, one-third peat moss, and one-third sterilized sod soil. When the seeds were subjected to low temperature [5° C. (41° F.)] prior to planting, the medium used was moist granulated peat moss.

When planted in the greenhouse seeds of *Caragana*, *Ligustrum ibota*, *Salvia*, and the soft seeds of *Hibiscus*, okra, and *Robinia* all germinated within 10 to 21 days, while seeds of *Rhamnus* and *Syringa* required about six weeks for total germination. Seeds of *Lonicera* and of *Ligustrum Regelianum* germinated within several months when held at 20° C. in moist peat moss. Seeds of a great many of the members of the Pinaceae family germinated slowly in the greenhouse but in many instances much more rapid and higher total germinations were obtained when the seeds, prior to planting, were subjected to low temperature for one or two months depending upon the species. Seeds of *Berberis*, *Liquidambar*, *Liriodendron*, and *Orixa* also produced better germination after a brief period at 5° C. In the case of *Arbutus*, *Cornus*, *Juniperus*, Mazzard cherry, *Pyrus*, *Rhus*, and *Rosa* the low temperature pretreatment was essential for germination. The period required at 5° C. varied from six weeks for *Pyrus* to three months for *Cornus*. *Rhus* seeds which also required three months at 5° C. were pretreated prior to the period at low temperature with concentrated sulfuric acid for not only were the embryos dormant but the coats were extremely hard. While an attempt was made to obtain maximum germination with each species not all possible combinations of temperature and periods of time were tested.

The results obtained with the germination tests and the rapid viability method of determining the germinative capacity of seeds are shown in the tables.

RESULTS

The data obtained in studies with 19 families and 58 species are presented. The 92 samples tested represent various types of seeds as well as various degrees of viability.

As is seen in Tables I and II rather close agreement was obtained with many of the samples when the results with the rapid viability method were compared with the germination tests. In nine samples (10 per cent) the results were identical, and with 30 of the samples (33 per cent) the results with the rapid viability tests were below (average 4.87 per cent, range 1 to 18 per cent) those obtained in the germination tests. That 53 of the samples (57 per cent) gave readings higher (average 8.17 per cent, range 1 to 31 per cent) in the rapid viability method than the germination results is not surprising for while attempts were made to obtain maximum germination not every possible combination of temperature and periods of time

TABLE I

COMPARISON OF THE TWO METHODS OF DETERMINING THE GERMINATIVE CAPACITY OF VARIOUS SEEDS

Family	Species	Crop	Experiment started, date	Results, % viable†	
				Rapid viability method	Germ. method
Anacardaceae	*Rhus aromatica* Ait.	?	Nov. 1945	91	93
Berberidaceae	*Berberis crataegina* DC	1946	Dec. 1946	79	81
	“ *Poiretii weichangensis* Schneid.	“	“ “	74	77
	“ *Thunbergii* DC	“	Jan. 1947	94	93
	“ “ *atropurpurea* Chenault	?	Mar. “	79	78
	Mahonia japonica DC	?	Jan. “	0	0
Caprifoliaceae	*Lonicera dioica* L.	1946	Dec. 1946	90	80
	“ *Korolkovii* Stapf	“	July “	84	77
Celastraceae	*Celastrus scandens* L.	1946	Jan. 1947	96	85
Compositae	*Silphium laciniatum* L.	1945	Nov. 1945	85	70
Cornaceae	*Cornus florida* L.	1946	Jan. 1947	57	63
	“ sp.?	“	“ “	90	84
Elaeagnaceae	*Elaeagnus reflexa* Morr. & Decne.	1946	July 1946	90	61
Ericaceae	*Arbutus Menziesii* Pursh (Madrona)	1942	April 1943	87	91
Hamamelidaceae	*Liquidambar styraciflua* L.	1941	Jan. 1943	75	78
	“ “ “	“	“ 1944	78	71
Labiatae	*Salvia officinalis* L.	1941	Dec. 1941	32	35
	“ “ “	“	“ “	10	8
	“ “ “	1943	Sept. 1943	82	82
Leguminosae	*Caragana arborescens* Lam.	1946	Oct. 1946	94	81
	Robinia PseudoAcacia L.	1938	Jan. 1945	45	39
	“ “ “	1940	“ “	80	75
Magnoliaceae	*Liriodendron Tulipifera* L.	1942	Jan. 1943	5	13
Malvaceae	*Abelmoschus esculentus* Moench (okra)	1944	Feb. 1946	95	88
	“ “ “ “	1945	“ “	63*	32
	Gossypium hirsutum L. (cotton)	?	April 1948	95	94
	“ “ “ “	?	“ “	80	71
	Hibiscus syriacus L.	1946	Jan. 1947	98	98
Oleaceae	*Ligustrum Ibota* Sieb.	1946	Jan. 1947	86	84
	“ “ “	“	“ “	90	75
	“ *Regelianum* Koehne	1945	May 1946	86	81
	Syringa vulgaris L.	1946	Jan. 1947	78	71
Rhamnaceae	*Rhamnus japonica* Maxim.	1946	Jan. 1947	99	96
Rosaceae	*Prunus avium* L. (Mazzard cherry)	1944	Oct. 1944	58*	44
	“ “ “ “ “	1946	Sept. 1946	34	20
	“ “ “ “ “	“	“ “	76	71
	Pyrus Calleryana Decne.	?	Nov. 1945	98	93
	“ *serotina* Rehd.	?	Sept. 1941	93	90
	Rosa multiflora Thunb.	1946	Feb. 1947	31	37
	“ *rugosa* Thunb.	“	“ “	44	46
Rutaceae	*Orixa japonica* Thunb.	1946	Dec. 1946	94	95
Vitaceae	*Parthenocissus tricuspidata* Planch.	?	Jan. 1947	46	64

* Many sluggish.
† Averages of duplicate tests.

were tried for every kind of seed. Also, in most instances, the percentage germination represented actual seedling emergence in soil which would be expected to be lower than results obtained in a germinator.

TABLE II

COMPARISON OF THE TWO METHODS OF TESTING THE VIABILITY OF PINACEOUS SEEDS

Species	Crop	Experiment started, date	Results, % viable†	
			Rapid viability method	Germ. method
Abies amabilis Forb.	?	Feb. 1947	54	28
" *balsamea* Mill.	?	Nov. "	34	38
" " "	1944	May 1946	5	4
" *concolor* Lindl. & Gord.	?	Jan. "	24	30
" *grandis* Lindl.	1945	Feb. "	40	36
" *magnifica* A. Murr.	?	Nov. 1942	0	0
" *Nordmanniana* Spach	?	Jan. 1947	18	14
Chamaecyparis thyoides Brit.	?	Feb. 1942	2	1
Cryptomeria japonica Don.	?	Jan. 1947	5	10
Juniperus virginiana L.	1940	Jan. 1943	5	1
" " "	"	" "	20	30
" " "	1943	" 1945	52	44
" " "	1944	" "	73	63
Larix europaea DC.	1940	Jan. 1945	24	26
" " "	?	April 1947	25	16
" " "	?	" "	43	43
Picea canadensis BSP.	1936?	May 1941	64	66
" *excelsa* Link	1940	Jan. 1945	52	43
" " "	1943	" "	84	83
" " "	?	April 1946	44	31
" *glauca* (*P. canadensis* BSP. var. *glauca*)	?	Sept. 1947	38	29
" *pungens* Engelm.	1942	Dec. 1944	25	25
" " "	1946	Jan. 1947	87	93
" " "	?	Nov. "	66	73
" *sitchensis* Carr.	?	July 1946	35	35
Pinus caribaea Morelet (slash)	1940	Dec. 1940	90	71
" " " "	1946	Jan. 1947	84	85
" *Jeffreyi* Balfour	?	Jan. 1946	5	8
" *palustris* Mill. (longleaf)	1946	Feb. 1947	69	77
" *ponderosa* Douglas	1939	Jan. 1945	4	8
" " "	1946	April 1947	19	19
" *resinosa* Ait.	1940	April 1945	78	77
" " "	1941	" "	88	81
" *sylvestris* L.	1940	Jan. "	93	87
" " "	?	Mar. 1947	86	68
" " "	?	" "	75	77
" *Taeda* L. (loblolly)	1939	Jan. 1943	59	65
" " " "	"	Dec. 1945	9	2
" " " "	1941	Jan. 1944	59	68
" " " "	1943	April "	57	55
" " " "	1946	Feb. 1947	74	72
Sequoia gigantea DC.	?	Nov. 1947	13	9
Taxodium distichum Rich.	1942	Jan. 1944	9	7
" " "	1944	" 1945	44	40
Thuja occidentalis L.	1941	June 1943	0	0
" " "	1943	Dec. 1944	1	9
" " "	?	Feb. 1945	16	6
Tsuga canadensis Carr.	?	Oct. 1941	39	25
" " "	?	Nov. 1942	1	3
" " "	?	" 1943	16	17

† Averages of duplicate tests.

In some cases when the percentage found viable by the rapid viability method was high, sluggish development of the embryos was noted (Table I—okra and Mazzard cherry). Since some of these seeds with low vigor may or may not germinate especially in soil it is extremely important in this test to observe differences in degree of vitality. Seeds in various stages of degeneration may have the entire embryo viable or parts of the embryo may be dead. These differences are frequently detectable in the rapid viability test as exhibited by the type of embryo development ranging from vigorous to very sluggish behavior or parts of the embryo may deteriorate. With seeds of some species it is not possible to detect such differences for even the best embryos show no development and all embryos showing no deterioration must be classified as viable. The types of hypocotyl development and cotyledon enlargement exhibited in various kinds of viable embryos have been described (10, 23, 30).

The data presented in Tables I and II in this study were combined with data (9, Table I, p. 291; 10, Table I, p. 342; 11, Table I, p. 457) previously published by the author. These combined data represent results with 175 samples covering 21 families and 87 species. In each case the percentage difference between results with the rapid viability test and germination method was calculated. As seen in Table III, 55 per cent of the cases were either identical or deviated up to 10 per cent when the two methods, using the germination results as the standard, were compared while a total of 80 per cent of the tests deviated from 0 to 30 per cent. It is interesting to note that the greater deviations occurred in the samples containing seeds of lowered vitality. Also, percentage differences become larger in these poor seeds as for instance with *Taxodium* (Table II) this difference was 29 per cent when 7 per cent germinated and 9 per cent were viable by the rapid viability test while the difference was only 10 per cent when 40 per cent germinated and 44 per cent were viable by the rapid viability test.

TABLE III

COMPARISON OF RESULTS BETWEEN RAPID VIABILITY AND GERMINATION TESTS*

Number of samples	Per cent germination	Percentage difference between results of germination and rapid viability tests														Coefficient of correlation
		0%		1–10%		11–20%		21–30%		31–50%		51–70%		>70%		
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
48	81–100	2	4	38	79	8	17									.9490
50	61–80	1	2	24	48	12	24	6	12	7	14					
22	41–60	1	5	8	38	4	18	3	14	3	14	2	8	1	4	
19	21–40	2	11	4	20	7	36			3	16	1	5	2	11	
36	0–20	12	33	2	6	1	3	4	11	4	11	3	8	10	28	
175	0–100	18	11	76	44	32	18	13	7	17	10	6	3	13	7	

* See text p. 235 for details of data.

A statistical analysis was made of the results from which the data in Table III were obtained. The coefficient of correlation was obtained from the values for $SPxy$, SSx, and SSy computed by the method illustrated by Denny (8, p. 58). In these calculations the rapid viability results (y) were compared with the standard (x) which was the percentage which germinated. The highly significant correlation coefficient of .9490 was obtained for this large set of data.

DISCUSSION

The results have shown that the observation of the behavior of the excised embryos serves as a good test for determining the viability of seeds. The method requires no special equipment and has been tested on many kinds of seeds. It is a rapid test when compared with the months required for germination of seeds of many of the various trees and shrubs. While the time required to test the viability of these seeds is greatly reduced this method requires considerable care for the excision of the embryos. It would be of tremendous value to have some reliable means of rapidly determining the degree of viability which would not require the excision of embryos.

Various methods for rapidly determining seed viability have been reviewed (10, 11) and only those references not previously cited are briefly discussed below. The cutting test (23, 48) usually does not supply the information desired except in some cases (71) as it only gives the number of seeds containing embryos. Specific gravity (6, 61) is also not a reliable index of germination capacity. Nelson and Burr (50) reported that the electrical potential readings of soaked corn grains were correlated with viability while differences between weak and vigorous development could be detected by recording the electromotive force emitted by the seedlings (66). Loss of vitality in rice (5) and inferior grades of ryegrass (44) could be detected by observing the fluorescence of seeds.

Plasmolytic agents (42, 51) and various chemicals (10, 14, 32, 47, 52, 56, 57, 73) have been tested as possible indicators of seed viability. Staining with indigo carmine, methylene blue, etc. has also been extensively studied (19, 34, 49, 54, 67, 69)—in these instances dead seeds readily stain while viable seeds remain uncolored. In other staining methods the chemical is colorless and when reduced by viable tissue the compound becomes colored as is the case with selenium compounds (3, 15, 17, 31, 35, 36, 37, 38, 58, 59, 74) and tetrazolium salts (7, 13, 18, 39, 40, 41, 46, 55, 62). When staining with these chemicals many report close agreement when results were compared with actual germination. The embryos must be in direct contact with the chemical and difficulties arise in interpreting the various shades and areas of coloration. In a study of staining various types of seeds with tetrazolium salts (12), it was necessary to excise embryos and while significant correlation was obtained when results were compared with the rapid viability tests, the deviations in many of the individual tests were large and frequent.

TABLE IV

LIST OF SPECIES, WITH LITERATURE REFERENCES, WHICH HAVE BEEN TESTED BY THE RAPID VIABILITY METHOD

Plant family	Species	Reference number under literature cited
Amaranthaceae	*Gomphrena globosa*	23
Berberidaceae	*Berberis Thunbergii*	23, 30
Bignoniaceae	*Bignonia* (*Campsis radicans*)	11, 23, 30
Boraginaceae	*Cynoglossum amabile*	23
Caprifoliaceae	*Symphoricarpos racemosus*	11
Celastraceae	*Celastrus scandens*	23, 30
	Euonymous sp.	23
Compositae	*Coreopsis*	27
	Wyethia scabra	11
Cornaceae	*Cornus* sp.	23
Cucurbitaceae	*Cucurbita*, *Cucumis*, and *Citrullus* spp.	23
	Echinocystis	28
Elaeagnaceae	*Shepherdia argentea*	11
Eucommiaceae	*Eucommia ulmoides*	11
Euphorbiaceae	*Euphorbia marginata*	23, 24
Hamamelidaceae	*Liquidambar styraciflua*	23, 30, 70
	Hamamelis spp.	10, 23, 30
Leguminosae	*Cercis canadensis*	11
Lythraceae	*Lagerstroemia indica*	11
Magnoliaceae	*Magnolia acuminata*	23
Martyniaceae	*Martynia*	23, 30
Oleaceae	*Fraxinus* spp.	23, 30, 64, 70
	Chionanthus spp.	10, 11, 23, 70
	Mendora spp.	11
Pinaceae	*Abies concolor*	23
	Pinus spp. (13 species)	1, 10, 11, 23
	Pseudotsuga sp. (Douglas fir)	10, 11
	Tsuga canadensis	23
Rosaceae	*Chaenomelis* (*Cydonia japonica*)	11, 23, 30, 70
	Crataegus spp.	10, 11, 23, 30, 70
	Lyonothamnus floribundus	11
	Malus spp. Apple	10, 23, 26
	Prunus spp. (9 species)	
	Apricot	26
	Cherry	11, 23, 26
	Peach	9, 23, 26, 29, 30, 68
	Plum	11, 26, 30
	Pyrus sp. Pear	10, 11, 26
	Rhodotypos kerrioides	10
	Rosa spp.	23
	Sorbus aucuparia	10, 11, 23, 70
Sporganiaceae	*Sporganium eurycarpum*	64
Umbelliferae	*Trachymene* (*Didiscus caerulea*)	23, 28
Urticaceae	*Cannabis sativa*	10
	Ulmus americana	2
Verbenaceae	*Callicarpa purpurea*	11

Many studies of enzyme activity and its relation to seed viability have been made (4, 20, 21, 45, 53, 60, 63). In some cases catalase and peroxidase cannot be used as an index of viability since dead seeds contain these enzymes. The fatty acid number of seeds as a possible clue to viability has been studied (16, 33, 72) and it has been suggested (43) that constancy of internal pH value is an important factor in seed viability.

Most of the literature on this subject deals with non-dormant seeds while the experiments reported in this study represent various types of seeds with various degrees of viability. Most of the germination tests were actual seedling emergence tests in soil, as this type of testing usually gives a more accurate estimation of the true planting value of a given lot of seeds. These germination results were compared with the behavior of the excised embryos.

The New York State Seed Law (22) requires the approximate percentage germination to be stated on all labels of seeds of forest, fruit, shade, or other trees and shrubs which are sold, offered or exposed for sale for seeding purposes. This rapid viability method is used for an approximation of the germinative capacity of many of these seeds (25, 65). The literature pertaining to the various kinds of seeds which have been tested by this method of determining viability in seeds appears in Table IV. To date, species representing 31 families have been tested in this manner.

SUMMARY

Studies on the determination of the germinative capacity of seeds by observing the behavior of excised embryos has been extended to include 58 species representing 19 families. The techniques employed for the excision of the embryos are described. Various kinds of seeds with various degrees of viability were tested. Agreement between the rapid viability method and germination tests was close. In a statistical analysis of these data combined with those previously published by the author a highly significant correlation between the two methods was obtained.

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SEED VIABILITY TESTS WITH 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE

Florence Flemion and Harriet Poole

A germination test has been the usual method of estimating the planting value of seeds. But many seeds are difficult to germinate and with such kinds the germination test requires special equipment, various pretreatments, and frequently long periods of time. Rapid methods of determining viability in seeds by eliminating the germination test have been studied by various investigators and reviews of this literature have been presented (10, 11, 12).

Recently, a biochemical method using 2,3,5-triphenyltetrazolium chloride has attracted much attention. This compound is one of the few organic chemicals which is colorless in the oxidized form while the reduced form has an intense red or orange color. When reduced by living tissue in seeds presumably only the viable embryos should stain.

The use of this chemical as a reliable index of viability of seeds has been studied (see Review of literature) and in many cases a close agreement between staining tests and actual germination tests has been recorded. Most of the seeds tested in this manner represent groups which germinate rapidly such as the cereals, etc. In the data presented below many types were tested—seeds representing 17 families and 58 species with embryos ranging from the non-dormant type to the very dormant, requiring from two to six months of specific conditions before germination can occur. Dead embryos rarely stained while viable embryos often stained deeply and uniformly. However, various shades of coloring as well as the staining of various areas made it difficult to ascertain which embryos contained sufficient vigor to germinate and produce plants.

MATERIALS AND METHODS

The seeds were either collected in the vicinity of the Institute at Yonkers, New York or purchased from or obtained through the courtesy of commercial seedsmen. All tests were carried out with excised embryos. To determine the accuracy and consistency of results with the tetrazolium method, the germinative capacity was also determined by the rapid viability test.

Details for conducting the rapid viability test have been published previously (12). Briefly, it consists of placing excised embryos on moist

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filter paper in Petri dishes at room temperature and observing their subsequent behavior over a period of two to seven days, depending upon the species and type of embryo.

Duplicate lots of 50 seeds each were used for each staining test as well as for each rapid viability test. Since close agreement between duplicates was obtained for each test, averages are presented in the various tables. Upon excision, the final number of embryos was often less than the original number of seeds counted because of some worthless seeds. The percentage of empty and deteriorated seeds thus encountered when excising the embryos for the staining test is shown in the tables.

For the staining tests, a one per cent solution of 2,3,5-triphenyltetrazolium chloride was used. A small sample was obtained through the courtesy of Dr. C. O. Jensen of the Pennsylvania State College. All of the experiments reported in this paper were carried out with the chemical synthesized by Dr. Edward Prill of this Institute. Since this chemical is somewhat light-sensitive, the compound as well as all the solutions were kept in the dark at room temperature in well-stoppered containers. In order to insure freshness, solutions were kept no longer than a few weeks. In correspondence with Dr. Jensen he stated that it was easy to determine whether the tetrazolium salt had deteriorated for it gradually becomes yellow when deterioraton takes place. Using this as a criterion the material used in the present tests showed no signs of deterioration.

For the tetrazolium tests the embryos were placed in vials and covered with an excess of the staining reagent. Immediately after the tetrazolium salt solution was poured over the embryos, the vials were placed in small wire baskets, lined and covered with heavy black cloth to exclude light, and were kept at various temperatures for various periods of time. For the data presented in the tables below a 24-hour period at 20° C. (68° F.) in darkness was used. At the end of the treatment period as each lot was examined at room temperature the amount and type of staining was noted.

The percentage of viable embryos as obtained by the rapid viability test was compared with the various amounts and intensities of staining produced by the use of the tetrazolium solution. In some cases these results were also compared with germination tests. One hundred different lots of seeds covering 58 species representing 17 plant families were tested. The number of species tested within a family ranged from one to as many as 18 while the number of samples of a given species ranged from one to eight. Not only are different families and species represented but also a great range in seed viability is illustrated in the various tables.

RESULTS

In preliminary experiments when intact viable seeds were soaked in the tetrazolium solution many of the embryos showed spotty or no staining but when excised they stained more uniformly. Thus to overcome any coat interference all of the subsequent tests were carried out with excised embryos. Also, attempts were made to section intact seeds, but due to the various types of coats and embryos encountered it was not practical, so for the sake of uniformity in all the tests the embryos were excised. Wetting agents such as Hydrocide 10-X Special and Vatsol OT were tested. In these experiments none of these compounds appeared to accelerate color development. Various periods of time as well as concentrations of tetrazolium salt ranging from 0.0001 to 1.0 per cent were tested. The degree of staining varied with the concentrations with no staining occurring in the lowest concentration. Attempts were made to run rapid viability tests on the embryos subsequent to the tetrazolium treatment. The higher concentrations and especially one per cent were apparently deleterious for the embryos subjected to these concentrations subsequently deteriorated when placed on moist filter paper in Petri dishes at room temperature. Since viability determinations by the rapid viability method could not be made on embryos treated with the tetrazolium solution, separate lots of excised embryos were used for the rapid viability tests.

Seeds were treated with one per cent solutions for 2, 4, 6, 24, and 48 hours at 20° C. In some cases several hours were adequate for obtaining the maximum amount of staining but usually longer periods were better. A period of 24 hours at 20° C. was most satisfactory when many samples were being tested. Forty-eight hours was often too long since frequently a precipitate would then occur in the solution. Apparently this reduction was due to the presence of the embryos for vials containing only solutions were used as controls during various tetrazolium tests and at no time was a precipitate found in these controls. Other temperatures such as 30° and 40° C. were also tried and with these higher temperatures shorter periods of soaking were satisfactory.

When embryos were soaked in a one per cent solution of the tetrazolium salt the degree of staining and intensity of color varied greatly as shown in Tables I, II, III, and IV. The observation and recording of the results were sometimes difficult due to the great variation in the amounts and shades of staining. In regard to the area stained in a given treated embryo, three classes were arbitrarily chosen—entirely stained, at least three-fourths of the embryo stained, and spot staining. It was sometimes difficult to differentiate between the two latter groups. Each classification was subdivided into three groups according to the intensity of red color—deep, medium, or light. Here again, it was sometimes difficult to distinguish differences.

The readings of extent and intensity of coloration were made upon examination of the outside of the embryos. Some were bisected to observe the extent of penetration and usually the coloration inside was the same

TABLE I

RESULTS OBTAINED WITH RAPID VIABILITY TEST AND STAINING WITH 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE IN DETERMINING THE VIABILITY OF VARIOUS SEEDS

Family	Species	Rapid viability test			Tetrazolium test										
					Per cent of seeds giving color										Deteriorated or empty seeds, not tested, %
					Entire embryo stained			Most of embryo stained			Spot staining			No staining	
		% vigorous	% sluggish	Total % viable	Deep	Med.	Light	Deep	Med.	Light	Deep	Med.	Light		
Aceraceae	*Acer tartaricum* L.	90	0	90		95					5				0
Anacardiaceae	*Rhus aromatica* Ait.	100	0	100	54			43			3				0
Berberidaceae	*Berberis Thunbergii* DC	77	6	83	49	21	27								3
	" " "	92	4	96		13	48				36			1	2
	" " "	30	4	34	37			19			25			19	0
Bignoniaceae	*Campsis radicans* Seem.	0	0	0										100	0
	Catalpa speciosa Warder	0	20	20										100	0
Caprifoliaceae	*Lonicera dioica* L.	78	0	78		10	74							9	7
Celastraceae	*Celastrus scandens* L.	2	0	2	11						4	24	24	31	6
Cornaceae	*Cornus* sp.	73	3	76	48	16	16				15			5	0
Hamamelidaceae	*Hamamelis virginiana* L.	0	34	34										100	0
	" " "	0	60	60										100	0
	Liquidambar Styraciflua L.	0	21	21										100	0
Leguminosae	*Cercis chinensis* L.	100	0	100	55			42			2			1	0
	Robinia PseudoAcacia L. a	54	0	54	32	25	2				16	7		18	0
	b	88	7	95	50	25					20			5	0
	" " " a	65	6	71	9	37					42			12	0
	b	91	2	93	31	34		9	7		9	8	2		0
Malvaceae	*Abelmoschus esculentus* Moench (okra)	77	8	85		83	1		7		5	3		1	0
	Gossypium hirsutum L. (cotton)	81	14	95			95							1	4
	" " "	0	0	0										94	6
	" " "	63	17	80			64			12				16	8
	Hibiscus syriacus L.	96	2	98	100										0
Oleaceae	*Chionanthus virginica* L.	56	22	78	24	42	28			3	2			1	0
	" " "	0	2	2					3	7	13	40	4	33	0
	" " "	54	9	63	70			19		9				2	0
	Fraxinus americana L.	63	28	91	21			55			21			3	0
	Ligustrum acuminatum Koehne	56	20	76	13	36		18			20		2	2	9
	" *Ibota* Sieb.	46	28	74	15	7	55						7	2	14
Rhamnaceae	*Rhamnus japonica* Maxim.	44	0	44	100										0
Rutaceae	*Orixa japonica* Thunb.	1	0	1							3			41	56
	Ptelea isophylla Greene	39	0	39	19	20	51							3	7
Taxaceae	*Taxus* sp.	77	0	77		21	60							19	0
Vitaceae	*Parthenocissus quinquefolia* Planch. (Virginia creeper)	8	5	13								1	13	86	0

a = Permeable seeds; b = Hard seeds.

TABLE II

RESULTS OBTAINED WITH GERMINATION TESTS, RAPID VIABILITY TEST AND STAINING WITH 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE IN DETERMINING THE VIABILITY OF VARIOUS PINACEOUS SEEDS

Species	Germination test*	Rapid viability test			Tetrazolium test										
					Per cent of seeds giving color										Deteriorated or empty seeds, not tested, %
					Entire embryo stained			Most of embryo stained			Spot staining			No staining	
		% vigorous	% sluggish	Total % viable	Deep	Med.	Light	Deep	Med.	Light	Deep	Med.	Light		
Abies balsamea Mill.	35	34	0	34	37			15	2				6	6	34
Larix europaea DC	—	28	0	28	16	3	3	21				12		12	33
Picea excelsa Link	—	0	0	0										74	26
“ “ “	—	0	0	0										92	8
“ “ “	70	73	0	73	53	7		13			8			3	16
“ *glauca* (*P. canadensis* BSP var. *glauca*)	29	38	0	38	13	2		27			6			32	20
“ *pungens* Engelm.	—	4	0	4	8	2							2	87	1
“ “ “	70	75	5	80	83		1				2			8	6
“ “ “	73	71	0	71	69			8						5	18
Pinus Banksiana Lamb.	—	39	0	39	48	1	13	9			2	1		20	6
“ *caribaea* Morelet	—	11	1	12		2	32	15		2				38	11
“ “ “	64	65	2	67	46			30						4	20
“ “ “	72	86	0	86	93		1								6
“ “ “	—	92	0	92	88	3					1				8
“ *echinata* Mill.	—	40	2	42	28			21			4			37	10
“ “ “	—	72	2	74	51			16	4	4				4	21
“ *palustris* Mill.	—	18	20	38			3	19			67			4	7
“ *Strobus* L.	—	0	0	0										92	8
“ *sylvestris* L.	—	42	1	43	34						18			24	24
“ “ “	—	64	3	67	57			12			1		6	11	13
“ “ “	—	76	1	77	70						4	3	1	5	17
“ *Taeda* L.	46	45	2	47	32			22						4	42
“ “ “	—	16	3	19	13	2		10			3			24	48
“ “ “	—	8	2	10	3			7				3		55	32
“ *Thunbergii* Parl.	—	27	2	29	30	4		12					9	39	6
“ *virginiana* Mill.	—	65	0	65	75	4	1	13						2	5
“ “ “	28	23	2	25	7			68					9	9	7
Pseudotsuga Douglasi caesia Schwerin	—	0	0	0										92	8
“ “ *glauca* Mayr.	—	0	0	0										84	16
Thuja occidentalis L.	—	0	0	0										91	9
Tsuga canadensis Carr.	—	1	0	1	6		2			2			7	81	2
“ “ “	—	0	0	27	40		2	6					3	4	45

* Seedling emergence in greenhouse soil.

TABLE III

RESULTS OBTAINED WITH RAPID VIABILITY TEST AND STAINING WITH 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE IN DETERMINING THE VIABILITY OF VARIOUS ROSACEOUS SEEDS

Species	Rapid viability test			Tetrazolium test										
				Per cent of seeds giving color										
				Entire embryo stained			Most of embryo stained			Spot staining				
	% vigorous	% sluggish	Total % viable	Deep	Med.	Light	Deep	Med.	Light	Deep	Med.	Light	No staining	Deteriorated or empty seeds, not tested, %
Chaenomeles japonica Lindl. (Japan quince)	30	14	44	65		13				20			2	0
“ “ “ “ “	0	2	2										100	0
Cydonia oblonga Mill. (quince)	0	31	31									9	90	1
Malus communis DC	0	54	54	6		3	51		4	21		4	10	1
“ *floribunda* Sieb.	0	33	33										100	0
“ *Scheideckeri* Spaeth	0	32	32										100	0
Prinsepia sinensis Oliv.	24	41	65									100		0
Prunus americana Marsh. (plum)	30	0	30	1	16	3		26	19	5		11	19	0
“ “ “ “	0	13	13									57	43	0
“ “ “ “	98	0	98			100								0
“ *avium* L. (Mazzard)	97	1	98		99		1							0
“ “ “ “	0	9	9		7			43			45		5	0
“ *cerasifera* Ehrh. (Myrobolan plum)	5	6	11			12							88	0
“ “ “ “ “	36	0	36			89						3	8	0
“ *Mahaleb* L.	98	0	98		100									0
“ “ “	0	70	70				35			45		20		0
“ *Persica* (L.) Stokes (peach)	87	5	92	43	46		6			3			2	0
“ “ “ “ “	63	7	70		81		1		5			1	12	0
“ “ “ “ “	0	23	23	28								68	4	0
“ *virginiana* L. (chokecherry)	0	0	0										100	0
Pyrus sp. (Kieffer pear)	0	21	21	12	17	5	60	3		2			1	0
Rosa multiflora Thunb.	79	0	79	46	29	25								0
Sorbus aucuparia L. (European mt.-ash)	97	1	98	98			1		1					0
“ “ “ “ “ “	63	4	67	45	19	25				4			4	3
“ “ “ “ “ “	0	62	62		6	41							53	0
“ “ “ “ “ “	0	10	10			37							63	0

as that on the outer surface. When spots or larger stained areas occurred the embryos were carefully examined in order to be properly classified. The viable embryos were usually white when excised. However, in a few instances, such as *Berberis*, *Celastrus*, *Cercis*, *Orixa*, and *Rhamnus*, they were yellow or green instead of white and this made the subsequent reading of color and intensity more difficult.

In some cases there was good agreement between the rapid viability and tetrazolium tests. When the total viability was zero or near zero, there was little or no staining (Tables I, II, III, and IV). However, with viable embryos there was considerable variation in the amounts and shades of color. The question arose as to which types and degrees of staining should be included when rating embryos as viable by the tetrazolium test.

TABLE IV

RESULTS OBTAINED WITH RAPID VIABILITY TEST AND STAINING WITH 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE IN DETERMINING THE VIABILITY OF VARIOUS CROPS OF RHODOTYPOS KERRIOIDES SIEB. & ZUCC. SEEDS

Crop	Rapid viability method			Per cent of seeds giving color with tetrazolium									
	% vigorous	% sluggish	Total % viable	Entire embryo stained			Most of embryo stained			Spot staining			No staining
				Deep	Med.	Light	Deep	Med.	Light	Deep	Med.	Light	
1947	98	2	100	16	62	1	20			1			
1946	79	10	89	29	64	2			2	2			1
1945	75	9	84	26	30	1	6	36	1				
1944	76	12	88	37	48	4		11					
1943	0	60	60	28	4	1	3	24		30	1	3	6
1942	0	55	55	30		3	55			12			
1940	0	2	2										100
1938	0	3	3										100

With *Acer tartaricum* seeds (Table I), 90 per cent were viable by the rapid viability test while 95 per cent were entirely stained a medium red color. With *Cornus* when all shades of entirely stained embryos were combined there was agreement. With *Rhus aromatica* the two methods agreed when the embryos which were completely stained were combined with those in which at least two-thirds of the embryo stained. There was considerable spot staining in some of the embryos such as *Robinia* (Table I), *Pinus palustris* (Table II), and *Prunus Mahaleb* (Table III).

Occasionally, a considerably greater percentage of the embryos stained than were found to be viable by the rapid viability test such as for instance

TABLE V

COMPARISON OF RESULTS BETWEEN RAPID VIABILITY AND TETRAZOLIUM TESTS

No. of samples	Rapid viability test, % viable*	Tetrazolium tests									
		Staining standard	Percentage difference between results of rapid viability and tetrazolium tests								Coefficient of correlation
			0%— (results identical)		1–30%		31–70%		>70%		
			No.	%	No.	%	No.	%	No.	%	
45	61–100	Entire embryo stained, deep & medium color	1	2	24	53	9	20	11	25	.7134
29	21– 60		1	3	7	24	10	35	11	38	
26	0– 20		9	34	2	8	0	0	15	58	
100			11	11	33	33	19	19	37	37	
45	61–100	Entire embryo stained, all shades	0	0	31	69	11	24	3	7	.7949
29	21– 60		0	0	7	24	8	28	14	48	
26	0– 20		9	35	3	3	14	54	0	0	
100			9	9	41	41	33	33	17	17	
38	61–100†	Entire embryo stained, all shades	0	0	33	87	5	13	0	0	.8630
22	21– 60†		0	0	11	50	4	18	7	32	
40	0– 20†		19	48	1	2	1	2	19	48	
100			19	19	45	45	10	10	26	26	
45	61–100	Entire embryo & most of embryo stained, all shades	2	5	37	82	5	11	1	2	.8202
29	21– 60		2	7	7	24	5	7	15	52	
26	0– 20		10	38	1	4	1	4	14	54	
100			14	14	45	45	11	11	30	30	

* All of the embryos showing development were listed as viable.

† Only embryos showing vigorous development were considered as viable.

Rhamnus in Table I. No staining occurred in the Hamamelidaceae (Table I). Although quite a few of these embryos were viable they were, however, low in vigor.

When determining viability by the rapid viability method differences in vigor were frequently detected. The percentages of both the sluggish and vigorous types of development observed are presented in the various tables.

In order to gain some conception of the dependability of the staining method when the rapid viability method was used as a standard, the various combinations of area and shade of staining were reassembled and some of these classifications appear in Table V. The number of samples (column 1) with a particular type of staining (column 3) which showed various degrees of viability (column 2) by the rapid viability method are recorded. These are compared with the percentage of staining obtained. In 9 to 19 per cent of the cases, the viability test and the staining test agreed exactly (columns 4 and 5, lines 13, 9, 5, and 1 from the bottom). In a third to about a half of the tests the tetrazolium readings were from 1 to 30 per cent above or below the percentage viable obtained by the rapid viability test. In a considerable number of the tests the deviation between the two methods was greater than 30 per cent. For instance as in the case of embryos completely stained including all shades (Table V, line 9 from the bottom, columns 8 and 10) one-half of the 100 samples tested deviated more than 30 per cent above or below the rapid viability readings. These data are also presented as a scatter diagram in Figure 1 which illustrates the wide deviation of many individual determinations from the regression line.

In a statistical analysis of the data, the coefficients of correlation and linear regressions were obtained with the method used by Denny (7, p. 58). In these calculations the staining results (y) were compared with the standard (x) which was the percentage found to be viable by the rapid viability tests. This standard was assumed to be correct since it has been shown that this method (12) of estimating viability agrees closely with germination tests.

In one of the groups shown in Table V (bottom line) where 59 of the 100 tests agreed within 30 per cent or less of the percentage obtained by the rapid viability test, the correlation coefficient was .8202, while in the group which had only 44 cases in this category the coefficient was .7134. The best correlation in Table V was .8630 for embryos which had completely stained (all shades) when compared with only the vigorous type of embryo development obtained in the rapid viability tests. However, these data also show considerable deviation for in 36 cases the staining results deviated more than 30 per cent from the rapid viability test.

Under the conditions of these tests significant correlation was obtained for this large set of data but the deviations in many individual tests was so great that it made the reliability of a given tetrazolium test rather questionable. The statistical calculations were based on the supposition that the rapid viability test was without error since it was used as the standard for comparison. It can, however, deviate as shown in Table II when compared with seedling emergence tests in soil (see also citation 12).

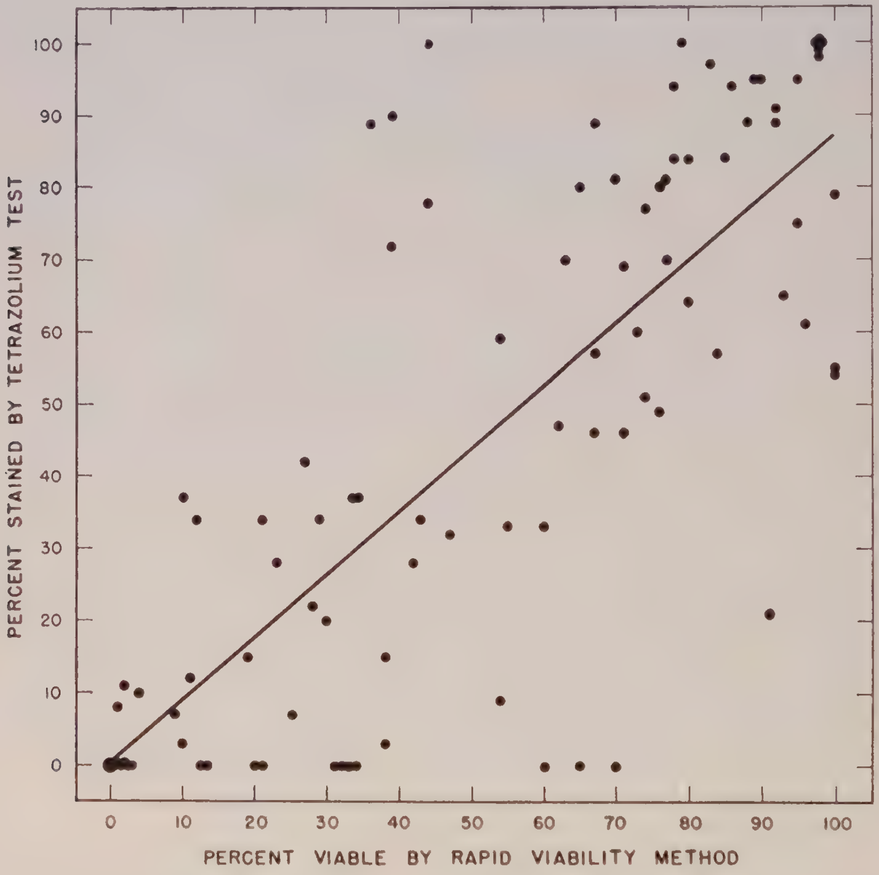

FIGURE 1. Correlation between the germination capacity of 100 samples of various seeds as determined by the rapid viability test and staining with 2,3,5-triphenyltetrazolium chloride (embryos completely stained counted as viable). The large circle in the lower left hand corner represents nine determinations in which the results for both tests were zero.

To obtain maximum germination in many of these dormant seeds very specific treatments for long periods of time are essential. And even when these exacting conditions are met other factors during germination such as media, soil organisms, temperatures, etc. may lower the percentage germination. Thus it is not surprising that when the rapid viability tests deviated from the germination results, the percentage viable was usually higher in the rapid viability tests (Table II). Because of the various factors which may affect germination, it seemed advisable to use the results obtained with the rapid viability test as the standard for comparing the staining results obtained with the tetrazolium test.

In regard to the tetrazolium tests various shades and amounts of staining of the embryos were obtained. The problem arose as to which types and degrees of coloration were correlated with viability. Upon examination of the data, the results of the staining test more nearly approached those obtained by the rapid viability test when all the embryos which stained completely were included regardless of the degree of staining or when in addition all embryos showing almost complete staining were considered viable. Any error in judgment regarding differences in color intensity was thus automatically eliminated by combining the various shades.

In the studies presented above the chances of the tetrazolium test differing as much as 70 per cent or more from the rapid viability test were quite high.

REVIEW OF LITERATURE

Many investigators have studied the use of various tetrazolium compounds for testing seed viability.

Kuhn and Jerchel (19), in studying the reduction of various tetrazolium salts by living tissue, kept *Lepidium sativum* seeds on filter paper moistened with one per cent solutions of 5-methyl- and 5-hendecyl-2,3-diphenyl salts. Normal germination was obtained but the leaves and various parts of the young seedlings were deep red.

Lakon (21) tested eight tetrazolium salts as to the possibility of determining viability of grain embryos. Two salts, 2,3-diphenyl-5-methyltetrazolium chloride and 2,3,5-triphenyltetrazolium chloride, were satisfactory. About eight hours were required to stain wheat, rye, and barley embryos while oats required 24 hours. Germinative capacity as determined by this method agreed with actual germination tests. With corn (22), the seeds after soaking in water about 18 hours were cut in half longitudinally and the cut surfaces submerged in one per cent 2,3,5-triphenyltetrazolium chloride. In about 24 hours the grains were examined and classified according to the extent of staining in the various regions.

Müller (25) used 2,3,5-triphenyltetrazolium chloride as a means of determining the viability of the endosperm from representatives of various plant genera. He found that seeds of *Agrostemma* and *Lepidium* were capable of germination in a one to two per cent solution while *Triticum* was not. Nadvornik (26) tested 2,3,5-triphenyltetrazolium chloride and 2,3-diphenyl-5-methyltetrazolium chloride as a vitality stain for seeds of fruit trees and shrubs and reported good accord when results were compared with the germination test. Cottrell (5) reported close agreement of the tetrazolium test with the standard germination method for seeds of cereals, peas, and vetches. He encountered various types of staining and indicated the types associated with normal germination. Latent cases of abnormality could be detected. Forward (13) treated seeds of various cereals. With oats, he obtained best results when soaked for 24 hours in a one to two per cent solution of 2,3,5-triphenyltetrazolium chloride while hull-less oats colored immediately.

Porter *et al.* (27) tested various samples of several members of the grass family, three leguminous species, buckwheat, and cotton. They encountered difficulties in making the necessary types of sections through the embryos and it was impossible to determine abnormalities such as baldheads. In a number of cases good correlation was obtained between the staining test and the standard germination tests. Shuel (28) removed the hulls of oats and barley and found it took about again as long a period for the unhulled seeds to stain. With wheat the tetrazolium test was reliable for fresh seeds but with older seeds (viability less than about 60 per cent) it was much less accurate as there was such great variation in the coloration which ranged from carmine to light pink. Correlation between lightly-staining and slowly-germinating seeds could not be found.

Goodsell (15) reported that the tetrazolium test could be satisfactorily used for making an early appraisal of minimal freeze damage to seed corn providing the grains contained less than 49 per cent moisture at the time the freeze occurred regardless of the amount of freezing. Franck (14) stated that the tetrazolium salt as well as other chemicals were being used in detecting the viability of various types of seeds.

It has been suggested that 2,3,5-triphenyltetrazolium chloride be used as a test reagent for the vitality of other tissues besides seeds. Kuhn and Jerchel (19) tested bacteria and yeast. Mattson *et al.* (24) used various parts of flowers, fruits, and vegetables, bull sperm, and blastoderm of hen's eggs. Waugh (33) tested the stem tissue of trees, shrubs, and cuttings. All of these animal and plant tissues reduce 2,3,5-triphenyltetrazolium chloride. Widely different substances serve as reducing agents for the tetrazolium salts such as zinc dust (19), fats (9), and enzyme systems, probably dehydrogenases (24).

Subsequent to Wieland's concept of the oxidation-reduction process within the living cell involving activated hydrogen which is released and a hydrogen acceptor which is reduced, Thunberg (30) named the enzyme group which activates hydrogen "dehydrogenase." He examined a large number of seeds in order to determine the ability of their extracts to oxidize (dehydrogenate) various substances and found that many seeds contain dehydrogenases. Seeds behave differently toward different donators and enzymes may possess different degrees of specificity in different seeds.

Dehydrogenases have been found in germinating seeds (3, 4, 6) as well as in dry seeds of legumes (2, 8), cereals (17, 18, 29), and many others (16, 30, 31). Seed coats of various *Prunus* species contained more dehydrogenases than did the embryos (16). Inactivity of some seeds is not due to lack of the enzyme but the coenzyme may be insufficient or not present (1). Dehydrogenases in moist seeds are more sensitive to temperatures than when the seeds are dry (20, 23). An exhaustive literature review of the dehydrogenases in seeds will not be presented here but the references just cited serve to illustrate that this enzyme group has been reported in many kinds of seeds.

In regard to enzyme tests for determining viability in seeds, good correlation with germination has been reported for many seeds with various enzymes but some enzymes such as catalase may be present long after the seed is dead. In such instances it is the ability of the seed to form new catalase that determines viability. If such a situation exists with the dehydrogenases the difficulty is probably in part overcome in the tetrazolium test by the soaking of seeds in water thus inducing the pre-germinating processes before treatment with the chemical.

In tests for dehydrogenase in wheat and pea seeds Turesson (32) found that the time for decolorization of methylene blue increased regularly with increasing age but that dead seeds also decolorized the solutions. Application of this particular technique is inadequate for determining whether any relationship exists between germination capacity and dehydrogenases in seeds. In staining tests for determining viability, methylene blue is also not very satisfactory because when reduced it is colorless. The oxidized colorless tetrazolium salts are unique by being reduced into the insoluble red formazans.

It will be interesting if the tetrazolium test can serve as a test for dehydrogenases in seeds. Triphenyltetrazolium chloride has also been used to locate the sites of reducing activity within the cells and as shown by Dufrenoy and Pratt (9) for culms of sugar-cane the reduction of this compound generally coincided with the location of oil droplets. It will not be surprising if the tetrazolium test cannot be used as a means of determining viability of all types of seeds for the chemical makeup of seeds of various species is quite different.

DISCUSSION

In the experiments described above it would have been an excellent test if the subsequent behavior of the tetrazolium-treated embryos could have been observed for various degrees of viability. But since this was impossible as the tetrazolium salt was apparently toxic, all the rapid viability tests were made on separate lots of embryos from the various respective samples. Within 25 hours after the embryos were excised, results were obtained with the tetrazolium test while two to seven days were required for the rapid viability test. Many of the seeds tested require from six weeks to six months of special treatment for germination to take place.

One of the difficulties encountered in the present tests was that frequently a precipitate appeared which in turn stained or speckled the embryos during the period the embryos were submerged in the tetrazolium solution. This phenomenon has been observed by others, for Kuhn and Jerchel (19) noted thick red specks deposited on yeast and bacteria from the solution and Porter *et al.* (27, p. 152) found coatings of reddish stain over the entire flat surfaces of the cotyledons of legumes when seeds had soaked in the solution for long periods.

As discussed above many investigators, working mostly with the cereals, have found close agreement when the tetrazolium test was compared with actual germination. However, before it can be recommended as a reliable index of seed viability by the technique used on the kinds of seeds tested in the present study much more detail needs to be worked out such as effect of temperature, treatment period, age, degree of dormancy, etc. Also a better understanding of its relation to respiratory processes and chemical makeup of the seeds should be known.

SUMMARY

A series of experiments designed to determine whether seed viability can be accurately estimated by the use of 2,3,5-triphenyltetrazolium chloride as a staining agent are reported. Dead embryos rarely stained while with viable embryos various types and degrees of staining were obtained. These staining results were compared with the percentage of embryos found viable by observing the behavior of the excised embryos (rapid viability method). In a statistical analysis of the data, significant correlation between the two methods was obtained, but large and frequent deviations were obtained in many of the individual tetrazolium tests. Many types of embryos ranging from the non-dormant to the very dormant as well as all degrees of seed viability were included in this study. One hundred different seed lots representing 17 plant families and 58 species were tested. Before the tetrazolium technique used in this survey can be recommended for any given species much more detailed work is necessary.

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GROWTH INHIBITORS IN SEEDS

LELA V. BARTON AND MARIE L. SOLT

INTRODUCTION

Inhibiting substances are known to be widely distributed in plant fruits and seeds. Juices of some fleshy fruits inhibit the germination of wheat even when the seeds are not in direct contact with the juice thus indicating the volatility of the inhibitor. Prolonged contact with the juice renders the seed incapable of subsequent germination (4). The inhibitor can be removed by adsorption by animal charcoal or by boiling. Chemically, three groups of substances appear to be responsible. First, essential oils such as are found in seeds of mustard, *Juniperus communis*, *Chenopodium ambrosioides* var. *anthelmínticum* and *Foeniculum vulgare* inhibit at high dilutions and when out of direct contact with the seed; secondly, alkaloids, the most active of which are cocaine, quinine, caffeine and physostigmine; and, thirdly, glucosides such as found in bitter almonds. It is suggested that the biological functions of these substances are to inhibit germination of seeds enclosed in their fruit. The absorbing power of the soil may partially inactivate these inhibitors. Stout and Tolman (18) attribute some of the effects of fruit juices to their osmotic pressure.

Inhibitors of both germination and enzyme activity have been found in a number of different seeds. Evidence has been presented that free ammonia released during germination from nitrogenous compounds of water extracts of the seed balls of sugar beets is an important factor in germination inhibition (18). These nitrogenous compounds are water soluble and are said to accumulate in the pericarp during seed development. This explained the beneficial effects of washing or soaking seeds prior to germination. Sufficient ammonia was released by enzymatic hydrolysis to inhibit the germination of naked sugar beet seed. A rise in pH did not account for the activity of the extract.

Inhibiting substances present in the mature *Iris* seed prevent the further growth of the embryo and keep the seed in a dormant condition according to Randolph and Cox (14). Embryos of ripe seed, when completely detached from the endosperm with which they are normally in contact, germinated promptly, but when left in contact with even a portion of the endosperm their growth was inhibited. Leaching with water usually increased germination. It is suggested that the substances are stable compounds relatively insoluble in water and not readily diffusible from the region of the embryo.

Auxin applied unilaterally to the tip of an *Avena* coleoptile results in a

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curvature away from the side on which the auxin is placed. An ether extract of radish cotyledons resulted in a curvature toward the side of the application, thus indicating the presence of an inhibitor (17).

Inhibiting substances not only affect the germination of seeds and subsequent growth of plants, but some of them also act as antibiotic agents. From aqueous extracts of finely-ground radish seeds (*Raphanus sativus*), Ivánovics and Horváth (8) isolated a liquid, raphanin, which, in addition to inhibiting germination and root growth of various seeds, gave a wide zone of inhibition on cylinder plates seeded with either *Staphylococcus aureus* or *Bacterium coli*. They presented evidence that the radish seeds do not contain the active principle proper, but rather that it is formed through an enzyme capable of converting an inactive precursor into an antibiotic. Extracts of intact seeds soaked in water were not active, indicating that the substances involved are bound to the cells. Lucas *et al.* (12) used aqueous extracts of macerated seeds of *Brassica* spp. and found that these crude emulsions contained some principle antagonistic to the growth of bacteria and fungi. These authors also stated that the antibiotic substance was not formed in the seeds but only in the process of their maceration with water.

References to these few reports on the subject will give the reader some idea of the type of work which has been done with some naturally-occurring growth inhibitors. Other references under "Results and Discussion" will relate more directly to the work to be reported below.

The experiments reported below were undertaken to determine whether the presence of an inhibitor or inhibitors in seeds was related to dormancy. A sensitive test object was found in the primary root of the wheat seedling. An adaptation of a method of growing wheat roots in solution to the problem at hand is described.

MATERIAL AND GENERAL METHODS

The growth of wheat roots in solutions containing the substance in question has been used as a measure of the presence of an inhibiting material. The technique used has been described previously (1) and is essentially as follows.

The three-salt solution was used as the control in most instances. It contained 0.02 M potassium acid phosphate, 0.02 M calcium nitrate, and 0.02 M magnesium sulfate. This solution has been found to be satisfactory for the growth of wheat roots (1). To the nutrient solution were added extracts, in different concentrations, of the seeds to be tested. For the most part, the extracts were made by adding distilled water to the ground seeds and allowing the mixture to remain at 30° C. for three hours, after which it was centrifuged and filtered. Variations in this procedure, as well as the porportions of water and ground seeds used for the extracts, will be given under "Results and Discussion" below. A distilled water control was added to each series.

To receive the solutions and the seedlings, 300 ml. Pyrex beakers, of the tall form and without lips, were covered with paraffined cheesecloth in which 25 small holes had been punched with a needle. A piece of paraffined thread was used to tie the cheesecloth securely over the beaker. The cheesecloth was then trimmed and the beaker was placed in a larger (600 ml.) beaker of the same form. The prepared solutions, distilled water and nutrient controls, and nutrient solutions containing different amounts of the extracts to be tested, were then poured into the beakers until the small one was entirely filled and the larger one was filled up to the top of the small one. Care was taken that no air bubble was left under the cheesecloth and that the level of the solution did not extend above the cheesecloth. Duplicate cultures of each solution were prepared.

The wheat (*Triticum* sp.) seeds[1] to be used were soaked in distilled water for three hours and then rinsed thoroughly. They were then placed furrow side down in 24-cm. glass moist chambers on filter paper which had been moistened with distilled water. It was necessary to take the seeds individually with forceps in order to place them with the furrow side down and far enough apart that the roots would grow out straight and free of the filter paper. From five to twelve moist chambers were used for each test, depending on the number of roots required. The chambers were left at room temperature until the wheat roots had attained a length of about 6 mm., usually about 28 hours. Seldom were seedlings longer than 7 mm. used for the tests, since the longer ones had secondary roots which hampered manipulation and increased the chances of injury in handling.

The germinated grains were placed through the holes in the cheesecloth so that the primary roots dipped into the solution. Each primary root was measured and the length recorded before placing in the solution. Then a root of the same length was placed in each of the other solutions in the series. This procedure was followed until each beaker contained 25 seedlings, each one of which had been measured. There were from 7 to 23 beakers in a series, and each series contained at least three controls, duplicate nutrient controls, and a single distilled water control. As a routine method, the extracts were tested in nutrient solutions, but the distilled water control served as a check on the water used to make the nutrient solutions and the extracts. The water was taken from a Barnstead steam still, the distilled water being delivered through block tin tubing.

The cultures were covered with watch glasses and placed in a dark oven at a temperature of 20° C. where they were allowed to remain for 64 to

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[1] The wheat seeds used throughout the tests were Marquis, 1946 crop, from the North Dakota Agricultural Experiment Station, Fargo N. D.

68 hours. The watch glasses were removed after the first 48 hours so as not to interfere with the growth of the coleoptiles. At the end of the growth period, the cultures were removed from the oven and the length of the primary root of each seedling was recorded. Also observations were made on the general appearance of the roots and tops and, in certain instances, the coleoptile length was also measured.

Unless otherwise noted, the growth values given in the tables were obtained by averaging the 25 measurements for each test solution, deducting the average initial length, and expressing this growth as a percentage of the average elongation for the nutrient controls.

Certain variations in this standard procedure will be noted below in the descriptions of the individual experiments.

The seeds from which extracts have been made for testing the presence of substances which might inhibit the growth of wheat roots are lettuce (*Lactuca sativa* L.), *Sorbus aucuparia* L., *Berberis Thunbergii* DC, bean (*Phaseolus* sp., Horticultural variety), wheat (*Triticum* sp. Marquis variety), barley (*Hordeum* sp.), edible soybean (*Glycine* sp.), eggplant (*Solanum melongena* L.), and tobacco (*Nicotiana tabacum* L., a Turkish variety). Freshly-harvested seeds of three varieties of lettuce, Big Boston, Grand Rapids, and Iceberg, of the 1947 crop were received on October 1, 1947, through the courtesy of Associated Seed Growers, Inc., New Haven, Conn.

For the tests reported in this paper a total of 112 cultures, or 2,800 wheat seedlings, were grown in the control nutrient solutions, and a total of 69 cultures, or 1,725 seedlings, in distilled water controls. The numbers of cultures of wheat roots to test the inhibiting effects of the various seeds were as follows: for *Sorbus*, 144; for *Berberis*, 65; for lettuce, 380; for bean, 200; and for the other non-dormant seeds combined, 74; making a total of 839 cultures. These added to the controls noted above involved the elongation and measurement of 25,500 wheat roots.

RESULTS AND DISCUSSION

Sorbus aucuparia. To determine whether very dormant seeds might contain an inhibiting substance preventing their germination, the seeds of the mountain ash were selected for study.

A seed collection was made at the Institute Arboretum in Yonkers, N. Y., on Sept. 22, 1947. The pulp was removed from the seeds which were then dried on blotters at room temperature. They remained in dry storage at room temperature until the inhibition tests were started in January, 1948.

The seeds of this species normally require an after-ripening period in a moist medium at low temperature before germination will take place (5). If the presence of an inhibitor in non-after-ripened seeds accounts for germination failure, then this substance should disappear with a treatment which breaks the dormancy.

To test this hypothesis, extracts were made of seeds which had been stored in moist granulated peat moss for periods of 2, 4, 8, and 11 weeks at 5° C. For controls, extracts were made of dry seeds and seeds soaked in distilled water at 25° C. for 16 hours at each of the testing periods. The second of these controls was for the purpose of testing a possible leaching effect. At the beginning of the experiment, the entire collection of seeds was divided into measured lots of 50 ml. each. The lots which were to receive the moist low-temperature treatment were wrapped in pieces of cheesecloth and placed in moist granulated peat moss. The pieces of cloth were large enough to permit spreading the seeds in a very thin layer, so that aeration would be adequate and mold would not appear.

At each testing period one lot, representing an initial volume of 50 ml., from each treatment (dry control, control soaked 16 hours at 25° C., and stored in moist peat at 5° C.) was ground in a mortar by hand. Thirty grams of washed quartz sand were added to each lot to facilitate grinding. A standard method and time was adopted for grinding in order to make the different tests comparable.

After grinding, the seed material was mixed with 250 ml. of distilled water, the temperature of which had been adjusted to 30° C., and the mixture was kept at this temperature for three hours. The material was then centrifuged and filtered and added to the nutrient solution at the rate of 8, 32, and 128 ml. per 1025 ml. and the wheat seedlings were introduced in the manner described above.

The resulting growth of the wheat roots is shown in Table I. Here, the growth obtained in duplicate cultures has been expressed as percentage of the growth in the nutrient control. It is evident that *Sorbus* seeds contain some material which inhibits the growth of wheat roots in solution. Extracts of the dry seeds, for example, permitted an average of about 29 per cent of the root elongation in the nutrient controls at a concentration of 8 ml. per 1025 ml. and much less in concentrations 4 or 16 times as strong. The same trend is to be seen for extracts of seeds soaked at 25° C. for 16 hours, and of seeds stored in moist peat at 5° C., though these appear to be less toxic (Table I). Also, extracts from seeds held at 5° C. may be less injurious than those soaked at 25° C. for 16 hours, indicating that under the former conditions more of the inhibitor disappears from the seeds. This cannot be attributed to an after-ripening at the low temperature, for the same effect is to be seen regardless of the length of time the seeds were at 5° C. After eight weeks at 5° C. germination was beginning in some of the lots, and yet extracts from non-germinated seeds of these lots were just as inhibitory in their action as those from seeds stored only two weeks at 5° C. An analysis of variance of the data in Table I confirmed the lack of effect of weeks of storage, and at the same time made clear the greater toxicity of the extract from dry seeds.

The tests were repeated after extension of the moist treatment at 5° C.

TABLE I

GROWTH OF WHEAT ROOTS IN SOLUTIONS CONTAINING EXTRACT OF SORBUS AUCUPARIA SEEDS

Weeks of storage	Seed treatment	Growth in different concentrations of seed extract (ml./1025 ml.), % of control		
		8	32	128
2	None (dry)	29	15	0
		31	19	0
	Soaked at 25° C. 16 hrs.	32	19	11
		37	21	12
	5° C. 2 wks.	31	29	13
		41	32	13
4	None (dry)	25	11	0
		27	9	0
	Soaked at 25° C. 16 hrs.	35	27	11
		33	28	10
	5° C. 4 wks.	47	27	16
		48	28	14
8	None (dry)	32	17	1
		32	20	1
	Soaked at 25° C. 16 hrs.	33	28	8
		33	28	8
	5° C. 8 wks.	46	16	3
		49	18	1

Analysis of variance

	D.F.	Mean S	F values
Total	53		
Weeks	2	6.7	0.141
Treatment	2	607.35	12.770*
Concentration	2	3743.15	78.703*
W×T	4	67.05	1.410
W×C	4	31.975	0.672
T×C	4	49.325	1.037
W×T×C	8	47.56	
Replicates	27	3.79	0.080

* Significant at the 1 per cent point.

to 11 weeks. It was found that wheat root elongation in the 8 ml. concentration of extract was almost identical with that obtained after eight weeks of treatment.

Since the 8 ml. concentration of the extract of all the *Sorbus* seeds, regardless of seed treatment, permitted less than 50 per cent of the growth of wheat roots in the nutrient control solution, lower concentrations were used at the 11-week storage period. This series included concentrations of

0.5, 1, 2, 4, and 8 ml. per 1025 ml. of nutrient solution. The result with the 8 ml. concentration has already been stated. With the 4 ml. solution increased root lengths were noted up to 73 per cent of the control. As the extract concentration was further decreased, less inhibition of root growth resulted, until 0.5 and 1 ml. showed no injurious effect as measured by wheat root elongation.

The appearance of four of the cultures at the end of the growth period is shown in Figure 1. These beakers were taken from the series run after eight weeks of pre-treatment at 5° C. The inferior growth in extract of dry seeds, 4, as compared with that of seeds pretreated at 5° C., 3, is shown.

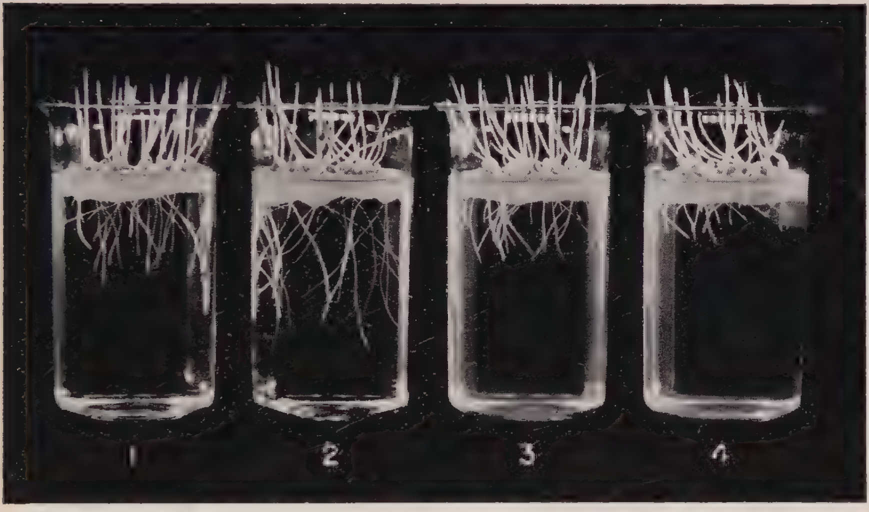

Figure 1. The growth of wheat roots in solutions containing: 1. distilled water; 2. three-salt nutrient solution; 3. three-salt nutrient solution+8 ml. water extract from *Sorbus aucuparia* seeds held moist at 5° C. for 8 weeks; and 4. three-salt nutrient solution+8 ml. water extract from dry *Sorbus aucuparia* seeds.

Also, the superior growth of the roots in the nutrient control, 2, over those in the distilled water control, 1, is seen. It will be noted that the coleoptile elongation has not been affected by the seed extracts (Fig. 1). In certain instances, notably the 128 ml. extract concentrations, shoot growth was reduced, however. If the root elongation was as little as 10 per cent or less of the nutrient control, the shoot elongation was reduced to approximately half of that of the control. When the roots failed to grow at all in the extract solution, or gave from 1 to 3 per cent of the control, the green tops of the seedlings still gave from 17 to 35 per cent of the control growth.

Berberis Thunbergii. Seeds of *Berberis Thunbergii* were selected for testing the presence of inhibiting substances because their germination, like that of *Sorbus*, is favorably affected by low-temperature pretreatment in a moist medium. Unlike *Sorbus*, however, *Berberis* seeds require a relatively short period of low temperature to prepare them for germination. Thus they may be considered less dormant, and, presumably, would contain less inhibiting substances if the latter proved, in any way, related to dormancy.

The procedure was the same as described above for *Sorbus* seeds. After seed samples had been in cheesecloth bags in moist granulated peat moss at 5° C. for one, two, or four weeks, extracts were made. At the same times, extracts were made of seeds which had been soaked in distilled water at 25° C. for 16 hours, and of the dry seeds. The resulting effect on wheat root growth in the presence of 0.5, 2, 8, and 32 ml. of such extracts per 1025 ml. of nutrient solution is shown for two of the series in Table II. It is apparent at once that *Berberis* seeds contain a more potent inhibitor of wheat root growth than those of *Sorbus*, since very little elongation (5 to 17 per cent of that of the nutrient control) occurred even at the 2 ml. dilution. A further reduction in concentration to 0.5 ml. permitted more growth.

TABLE II

Growth of Wheat Roots in Solutions Containing Extract of Berberis Thunbergii Seeds

Weeks of storage	Seed treatment	Growth in different concentrations of seed extract (ml./1025 ml.), % of control							
		0.5		2		8		32	
		a	b	a	b	a	b	a	b
2	None (dry)	—	—	12	14	5	4	3	2
	Soaked at 25° C. 16 hrs.	—	—	10	10	4	5	1	2
	5° C. 2 wks.	—	—	16	15	5	5	2	2
4	None (dry)	52	50	14	17	6	7	—	—
	Soaked at 25° C. 16 hrs.	32	28	5	5	5	5	—	—
	5° C. 4 wks.	44	49	10	14	6	6	—	—

In this case, there is apparently no leaching out of the injurious substance as might have happened for *Sorbus* seeds. Also, again, there seems to be no definite relation between the presence of wheat root growth inhibitors and dormancy. Samples of the *Berberis* seeds pretreated at 5° C. were planted in the greenhouse after one, two, and four weeks at the low temperature where 17, 26, and 60 per cent seedling production were obtained. These results indicated progressive after-ripening with increased time at 5° C.

It can be concluded, then, from the two examples of dormant seeds given above, that dormancy is not imposed solely by a substance extracted by water and deleterious to the growth of wheat roots in solution. This does not preclude the possibility of a substance not soluble in water or of another type of substance causing failure to germinate.

Lactuca sativa. We have already considered the growth-inhibiting substances present in two types of dormant seeds, *Sorbus* and *Berberis*. Both of these germinate better after pretreatment in a moist medium at low temperature. The seeds of the former are more dormant than those of the latter as shown by complete germination failure without low temperature pretreatment, and by the longer time period required for after-ripening at the low temperature.

Another type of dormancy exists in a number of seeds when they are freshly harvested but disappears upon dry storage. To test the possible relation of an inhibitor to dormancy in seeds of this category, freshly-harvested seeds of lettuce of the varieties Big Boston, Grand Rapids, and Iceberg were used. Experiments were started the first week in October on seeds which had been harvested the latter part of September.

That lettuce seeds contain some substance which leaches out and is capable of reducing their own germination has been shown by Shuck (15). He found that the placing of six consecutive lots of lettuce seeds in contact with the same moist substratum in which the water level was held uniform reduced the germination from 87 to 3 per cent. The accumulation of substances on the substratum included possible metabolic products of the germination process as well as those leached from the dormant seeds since some of the seeds germinated. However, a temperature above 20° C. and less favorable for the germination of dormant lettuce seeds caused the inhibiting substances to be more effective. Two thousand seeds of Big Boston at 20° C. were needed before germination was inhibited, while at 25° C. only 600 were needed. Black Seeded Simpson seeds showed only slight reduction of germination at 25° C. as compared to Big Boston, but at 30° C. there was a rapid formation of the inhibitor. Stout and Tolman (18) attributed the harmful effect to the liberation of free ammonia from Black Seeded Grand Rapids seeds which have a high nitrogen content. A water extract released twice as much free ammonia as was necessary to inhibit the germination of lettuce seeds.

If dormancy at harvest is due to a chemical inhibitor in lettuce seeds, and such dormancy disappears with dry storage, then the inhibitor should also disappear as the storage period lengthens. This premise was used as the basis for the tests reported here, where the growth of wheat roots in extracts of lettuce seeds was measured.

The dry, freshly-harvested seeds were ground in a Hobart burr mill. To 50 ml. of the seed meal were added 200 ml. of distilled water and the mixture was kept at 30° C. for three hours. The resulting extract was then added to the standard nutrient solution in concentrations of 2, 8, 32, and

128 ml. per 1025 ml., and wheat roots were introduced for growth effects. Results are shown in Table III.

That lettuce seeds contain some material which is injurious to wheat roots is obvious. As little as 2 ml. per 1025 ml. in nutrient solution allows only 78 to 80 per cent of the growth in the control for the Big Boston variety; 68 to 72 per cent for Iceberg; and only 57 to 69 per cent for Grand Rapids. This greater toxicity of the Grand Rapids extract in the 2 ml. concentration persisted for all testing periods. Increasing the amount of extract introduced into the nutrient solutions reduced the growth still further, but at the higher concentrations the difference in variety effects became less marked. This is shown more clearly in the graphs in Figure 2.

TABLE III

GROWTH OF WHEAT ROOTS IN SOLUTIONS CONTAINING EXTRACTS OF THREE VARIETIES OF LETTUCE SEED

Variety	Weeks of dry storage	Growth in different concentrations of seed extract (ml./1025 ml.), % of control							
		2		8		32		128	
		a	b	a	b	a	b	a	b
Big Boston	0	80	78	35	39	23	20	—	—
	1	84	70	40	46	29	26	—	—
	2	64	68	31	26	35	27	11	12
	4	60	75	28	38	24	23	14	16
	8	—	—	—	—	40	35	—	—
	19	88	86	51	52	38	35	17	14
	51	90	91	57	52	42	44	15	15
Grand Rapids	0	57	69	31	26	25	26	—	—
	1	61	62	22	25	14	19	—	—
	2	48	50	23	23	24	29	12	15
	4	48	62	21	21	21	16	14	17
	8	—	—	—	—	28	26	—	—
	19	59	59	39	38	34	35	14	14
	51	73	66	34	36	29	31	18	16
Iceberg	0	72	68	40	30	40	40	—	—
	1	79	83	36	42	31	31	—	—
	2	72	76	28	28	30	28	7	7
	4	73	72	27	26	27	27	11	9
	8	—	—	—	—	41	37	—	—
	19	82	80	50	52	30	45	12	14
	51	79	79	52	51	42	41	12	14

Both Table III and Figure 2 also show no significant differences in the injurious effect of the extract up to at least eight weeks of dry storage. At the eight-weeks' test for all three varieties at 32 ml. concentration only, there seemed to be a trend toward a decreased activity of the inhibitor. This trend became more pronounced after 19 weeks of dry storage as measured by the effect on wheat roots of the 8 and 32 ml. concentrations of the extracts. This parallels the decreasing dormancy of Grand Rapids and Ice-

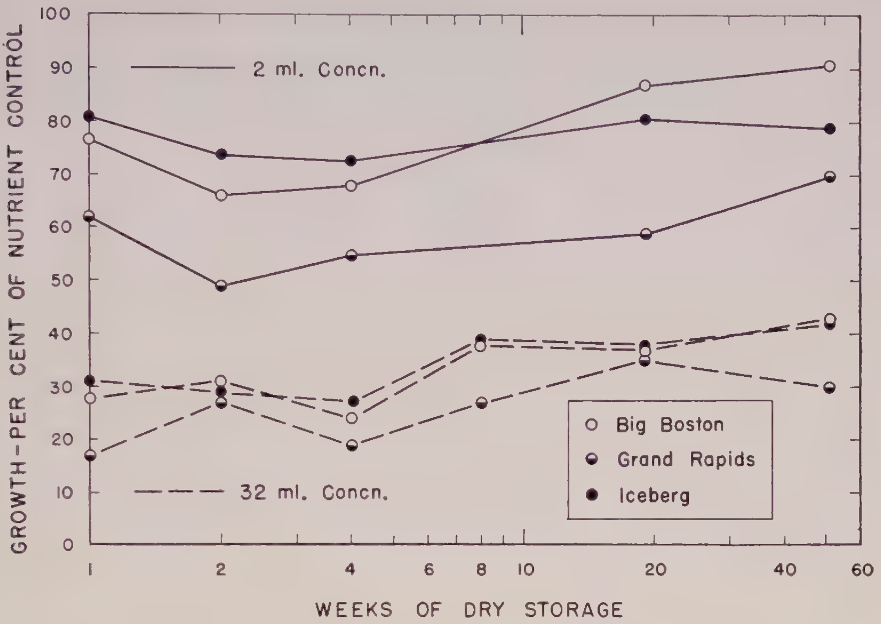

FIGURE 2. Effect of water extracts of three varieties of lettuce seed upon the growth of wheat roots in solution. Averages of duplicate cultures expressed as percentage of growth in the three-salt nutrient control.

berg varieties shown by the ability of the former to germinate at 25° C. and the latter at 30° C. (Table IV), but after 19 weeks of dry storage seeds of the Big Boston variety were still very dormant, requiring a temperature below 25° C. for germination. Results for both inhibitor and germination tests indicated only slight changes when the dry storage period of the seeds was extended from 19 to 51 weeks (Tables III and IV, and Fig. 2). Also, there is no clear relationship between the greater toxicity of the Grand Rapids variety mentioned above and the degree of dormancy of the seeds from which the extracts were made. According to the difficulty of seed germination (Table IV) Big Boston extract should have been most injurious to wheat roots.

Some of the same extracts made after two weeks of dry storage and used for wheat root inhibition were used to test their effects on germination. Each variety of seed was used with its own extract. The mixtures of extracts and nutrient solutions were used to moisten filter paper on which seeds were placed for germination at 20° C. Nutrient solution alone was used for the control lots. No effect on the germination of Grand Rapids and Iceberg seeds was noted. Germination of Big Boston seeds was reduced in all cases, including the controls. The nutrient solution alone permitted only 11 per cent germination.

To test further the possible inhibiting effect of Big Boston seed extract

TABLE IV

GERMINATION OF LETTUCE SEEDS FROM WHICH EXTRACTS WERE MADE; DUPLICATES OF 100 SEEDS EACH

Weeks of dry storage in lab.	Per cent germination on moist filter paper								
	Big Boston			Grand Rapids			Iceberg		
	20° C.	25° C.	30° C.	20° C.	25° C.	30° C.	20° C.	25° C	30° C.
Fresh	75	0	0	99	1	0	92	93	1
1	86	2	0	100	6	0	98	91	1
2	92	1	0	100	4	0	96	88	2
3	95	1	0	98	1	0	97	84	1
4	95	1	0	100	21	1	99	94	1
6	92	3	1	100	12	1	95	91	1
8	93	2	0	99	10	1	96	94	12
19	95	6	0	100	86	4	96	94	65
51	97	15	2	99	85	1	94	89	63

on germination, another experiment was set up after five weeks of dry storage. Distilled water was used instead of nutrient solution to dilute the extract and for the control cultures. Seeds of the three lettuce varieties were soaked in these solutions at both 20° and 30° C. for 16 hours, after which the solutions were drained off, but the seeds were not washed. They were placed immediately on filter paper moistened with tap water at 20° and 30° C. for germination. The results are shown in Table V. In spite of the fact that the extract concentration in one case was twice as great as the strongest one shown in Table III, no striking inhibiting effect on germination was noted. There may have been some reduction in germination of Iceberg seeds at 30° C. following a presoaking in 256 ml. concentration at 20° C.

Germination tests were also conducted with extracts made by soaking intact seeds of each variety (125 ml. of seeds +500 ml. of distilled water)

TABLE V

GERMINATION TESTS OF THREE VARIETIES OF LETTUCE SEEDS TREATED WITH EXTRACT OF BIG BOSTON SEEDS STORED DRY FOR 5 WEEKS

Soaking		Per cent germination of three varieties at 20° C. and 30° C.					
Extract Concn.*	Temp. (°C.)	Big Boston		Grand Rapids		Iceberg	
		20° C.	30° C.	20° C.	30° C.	20° C.	30° C.
0	20	89	9	98	12	99	43
	30	72	0	91	0	93	4
128	20	75	4	98	4	95	48
	30	71	1	96	1	94	2
256	20	87	2	100	1	91	22
	30	81	1	95	0	96	9

* Ml. per 1025 ml.

at 20° C. for five hours. The extracts were stored overnight at 20° C., then 8.5 ml. of each were added to a Petri dish containing five pieces of filter paper. Seeds of the three varieties were used both for the making of the extracts and for the germination tests. The germination of each variety was tested at 20° C. with extracts of each variety. No inhibitory effects were noted.

Extracts of intact seeds made by soaking 50 ml. of seeds in 200 ml. of distilled water at 20° C. for five hours, were used in nutrient solutions and wheat roots were introduced in the regular way. The elongation of the roots was reduced to approximately 65 per cent of the control in concentrations of 128 ml. per 1025 ml. and were somewhat shorter in twice that concentration regardless of the lettuce variety. It should be recognized, of course, that the wheat root growth test is much more sensitive than a germination test.

To test further any relationship between after-ripening of lettuce seeds and the activity of their extracts, the latter were given certain treatments known to affect lettuce dormancy. For example, it is known that freshly-harvested lettuce seeds may be induced to germinate at temperatures which are inhibitive by pretreatment at a low temperature in a moist condition. Also thiourea has been shown to break their dormancy under certain conditions (6). It was thought that if the activity of the wheat root inhibitor were related to degree of after-ripening, it might be affected by treatment of the extracts by low temperature or thiourea.

Storing the lettuce seed extracts at 5° C. for three days or two weeks failed to affect their activity. Limited tests with thiourea, on the other hand, indicated a possible deactivation of the inhibitor in extract of Big Boston lettuce. This extract was used in the regular way in a concentration of 8 ml. per 1025 ml. of nutrient solution and to the cultures were added 0, 10, 20, 40, 80, and 100 ml. of a 1 per cent solution of thiourea. Wheat roots in these cultures gave 48, 85, 76, 63, 56, and 56 per cent of the nutrient control growth respectively. Thus 10, 20, and 40 ml. of the thiourea solution seemed to have an antagonistic effect on the inhibitor in the lettuce extract, permitting greater elongation of wheat roots in solution.

Adding as much as 80 or 100 ml. of the thiourea solution introduced a harmful effect of the thiourea itself. In nutrient control solutions without any lettuce extract, adding 10, 20, 40, 80, or 100 ml. of 1 per cent thiourea, gave 107, 92, 78, 69, and 65 per cent respectively of the wheat root lengths of the nutrient control. Thus it is seen that while 10 ml. of the thiourea solution actually appeared to stimulate growth of wheat roots, the larger amounts reduced the growth. In no case did the addition of thiourea to the solutions containing the lettuce extract permit as much wheat root growth as the corresponding thiourea control. Walti (19) found that the germination of lettuce seeds which had been inhibited by an extract of kale seeds, could be reactivated upon the addition of thiourea or thioacetamide. These results point to a promising line of investigation which will be pursued further in later tests.

Distilled water instead of nutrient solution was used as a carrier for the lettuce extracts in an experiment tried after the lettuce seeds had been stored dry for six weeks. The comparison of the growth of wheat roots in these solutions is shown in Table VI. In the 8 ml. concentration, the extracts in distilled water allowed more growth than in nutrient solution. At the higher concentration of 32 ml. root growth was approximately the same in distilled water and nutrient solution. The explanation for this is not clear. If it is just a question of supplying the wheat roots with some food which is not present in the distilled water, there should be four times the amount of food in the 32 ml. concentration, but there was less growth here than when only 8 ml. of the seed extract was supplied. There is a possibility, of course, that at the 32 ml. concentration the favorable effect of extra food for the roots is masked by the extra amount of the inhibitor present. It is recognized that the seed extracts are very complex chemically and might very well contain both food and inhibitors.

TABLE VI

GROWTH OF WHEAT ROOTS IN EXTRACTS OF THREE VARIETIES OF LETTUCE AFTER 6 WEEKS OF DRY STORAGE

Variety	Medium	Per cent of nutrient control						Per cent of distilled H_2O control					
		8 ml. in 1025		32 ml. in 1025		No extract		8 ml. in 1025		32 ml. in 1025		No extract	
		a	b	a	b	a	b	a	b	a	b	a	b
Big Boston	Nutrient	36	45	42	41	100	100	64	79	74	71	179	174
	Distilled H_2O	70	68	45	41	57	57	124	119	79	71	100	100
Grand Rapids	Nutrient	40	34	36	36	100	100	68	59	61	61	161	178
	Distilled H_2O	69	69	39	43	59	57	117	117	66	73	100	100
Iceberg	Nutrient	46	46	41	44	100	100	78	78	71	76	161	178
	Distilled H_2O	70	63	46	40	59	57	120	107	78	68	100	100

Pole bean, Horticultural variety. A number of experiments were performed using beans as an illustration of non-dormant seeds and testing the toxic effects of the extracts of these seeds on the growth of wheat roots. The extracts were made in the proportion of 25 grams of beans made up to 200 ml. with distilled water except where otherwise noted. The extracts and their fractions reported in this section were prepared by Dr. Edward A. Prill of this Institute. The beans were first ground in a Hobart burr mill and then in a Wiley mill with a 60-mesh sieve.

An extract made by treating the bean meal with distilled water for three hours at 30° C. proved toxic to the growth of wheat roots (Table VII, Exp. 1). Autoclaving this extract for 20 minutes at 15 pounds' pressure or boiling for 30 minutes failed to destroy the inhibitor. Also, heating the bean meal on a steam bath at 90° C. for two hours did not appear to lessen the retardation of wheat root growth (Table VII, Exp. 2).

Bean meal (25 g.) was ashed and the ash treated with nitric acid, filtered, evaporated, and then made up to a 200 ml. solution containing the soluble nitrates of the ash. Wheat root elongation was stimulated in the 2 and 8 ml. concentrations, and remained about the same as the control in the 32 ml. concentration, but almost no growth took place in the 128 ml. solution (Table VII, Exp. 2). The inhibition of this last solution probably was due to the high acidity (pH 3.2).

TABLE VII

GROWTH OF WHEAT ROOTS IN SOLUTIONS CONTAINING BEAN SEED EXTRACT

Experiment No.	Bean extract	Growth in different concentrations of seed extract (ml./1025 ml.), % of control							
		2		8		32		128	
		a	b	a	b	a	b	a	b
1	H_2O extract untreated	77	87	60	56	17	17	12	10
	Autoclaved 20 min. 15 lbs.	—	—	59	55	33	31	—	—
	Boiled 30 min.	—	—	59	56	26	28	—	—
2	Beans heated in steam bath 90° C. 2 hrs.	87	88	58	62	21	21	21	23
	Soluble nitrate from bean ash	111	104	108	113	93	93	1	1
3	Water fraction from H_2O chloroform partition	78	75	49	51	18	18	11	11
	Chloroform fraction from H_2O chloroform partition	91	93	91	88	81	65	73	65
	Dialyzate	62	65	42	48	65	64	1	4
	Non-dialyzable residue	82	83	56	55	50	54	1	0
4 (pH adjusted to 4.3)	"Phospholipide" fraction from $CHCl_3$ extract	93	89	75	74	73	68	54	54
	Ppt. from methanol extract	111	104	93	104	71	73	31	31
	Soluble part of methanol extract	55	53	27	26	24	23	14	14
	H_2O extract	70	76	46	46	27	29	14	14

Dialysis of a water extract of bean meal through a cellophane membrane with agitation for three hours did not effect a separation of the inhibitor since both the dialyzate and the non-dialyzable residue reduced wheat root growth (Table VII, Exp. 3).

In still another test, a water extract was shaken with an equal volume of chloroform. Each of the two layers from this partition was freed of chloroform and converted into an aqueous solution. It will be noted in Table VII, Exp. 3, that the water fraction was more toxic throughout than the chloroform fraction. This effect is also shown in Figure 3.

Determinations of the pH of the solutions in both the outer and inner

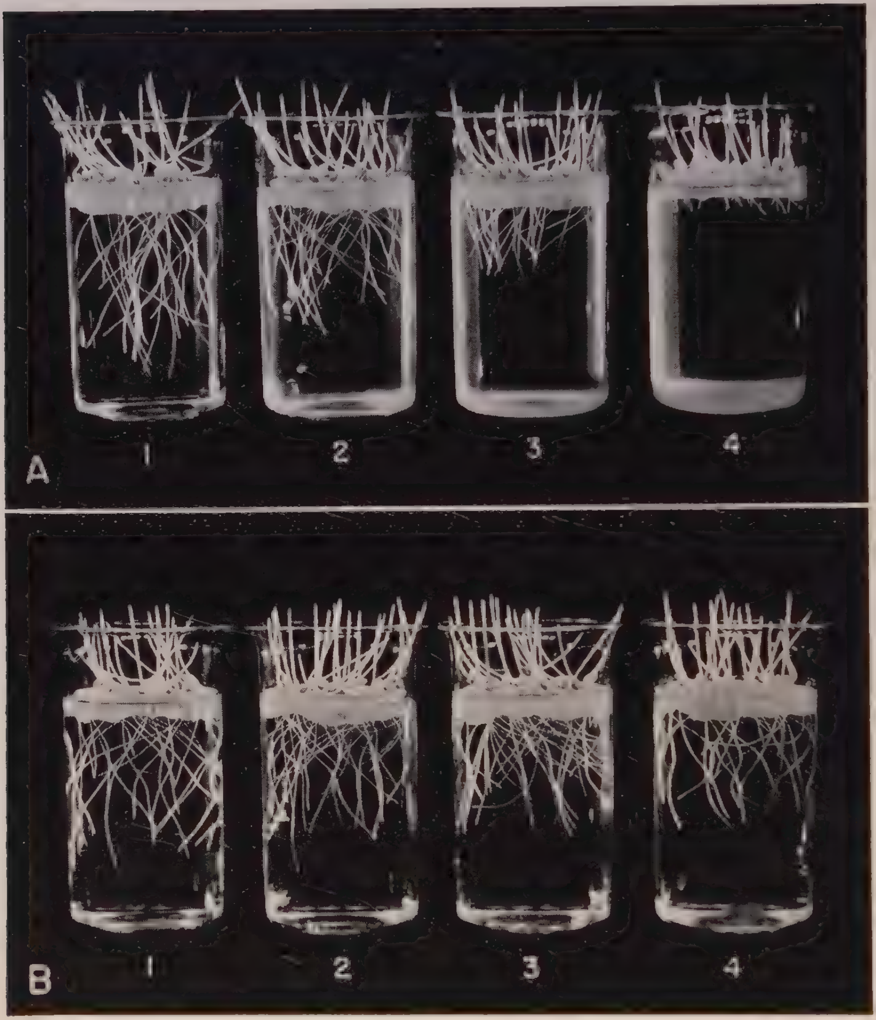

FIGURE 3. The growth of wheat roots in three-salt nutrient solution containing extracts from bean seeds: A. water fraction, and B. chloroform fraction from water-chloroform partition of the extract in concentrations of 0, 2, 8, and 32 ml. per 1025 ml. (1, 2, 3, 4).

beakers used in Exp. 3, Table VII, were made at the termination of the tests. It will be recalled that the roots grew in the inside beaker in each case, while the solution in the outside beaker had no contact with the wheat seedlings. The results should show, then, the effect of the roots on the pH of the medium in which they grow, and the possible contribution of acidity to the injury of the roots. The solutions did not vary greatly in their reaction at the beginning of the test as indicated by the pH determinations (range, 4.0 to 5.0) on the solutions in the outer beaker. The growth of the roots in the solutions tended to make them slightly less acid in most cases as shown by the readings on the solutions in the inner beakers. However, the pH range (4.0 to 5.2) of these solutions was almost the same as noted above. This change is probably not significant as far as reduction of root elongation is concerned.

Exp. 4, Table VII, deals with the testing of solutions obtained by extracting 100 grams of bean meal successively with the following series of organic solvents: petroleum ether (B.P. 30° to 60° C., extract not tested), chloroform, and anhydrous methanol in a Soxhlet extractor, and extracting the residue with water in the usual manner. From the chloroform extract, a fraction insoluble in ethyl ether and acetone mixture, possibly a phospholipide, was separated. The methanol extract was separated into two fractions, namely, the solid which collected on the walls of the extraction flask and the clear methanol solution of more soluble substances. Each fraction was freed of any organic solvent present by evaporation under reduced pressure, taken up in water, adjusted to pH 4.3 with sulfuric acid and made up to a volume of 200 ml. The growth of wheat roots in these solutions is shown in Table VII, Exp. 4. It should be pointed out that the water extract in Exp. 4 was not the same as that in Exp. 1, since four times as much bean meal per 200 ml. was used and some substances had been removed by previous extraction with organic solvents.

It was of interest to determine what part of the bean seed contained an inhibitor for wheat root growth. Seed coats, cotyledons, and embryo axes (consisting of plumule, hypocotyl, and radicle) were separated and 2 grams of each were ground by hand in a mortar and extracted with 160 ml. of distilled water at 30° C. for three hours. These extracts were added to nutrient solutions in the regular way, in concentrations of 2 and 4 ml. per 1025 ml. With 2 ml. of the extracts of seed coats, cotyledons, and embryo axes, wheat roots grew to 48, 73, and 59 per cent of the nutrient controls. With the amount of extract added to the nutrient increased to 4 ml. for each culture, the growth of the roots was 30, 59, and 47 per cent of the nutrient control for extracts of seed coats, cotyledons, and embryo axes respectively. It will be noted that, per unit weight, seed coats contained most inhibiting substances, and cotyledons least. For a 2-gram sample, coats from 75 seeds, cotyledons from 4-1/2 seeds, and embryo axes from 300 seeds were required.

Often upon addition of the extract to be tested to the nutrient solution a precipitate was formed, making the test solution more or less turbid. This brought up the question of the suitability of the nutrient solution as a carrier of the extracts, in spite of the demonstrated favorable effect of the nutrient itself. To get further information on this subject distilled water, tap water, the regular 0.02 M three-salt solution, and a dilution of the latter to one-sixteenth of its regular strength were used with 0, 8, and 32 ml. of water extract of bean meal per 1025 ml.

The growth of wheat roots in these solutions is shown in Table VIII. Duplicate beakers have been averaged to get these figures. Thus each figure represents measured lengths of 50 instead of 25 seedling primary roots. When the growth is expressed as percentage of the nutrient control growth, it will be seen that tap water alone allows a growth of over 100 per cent. However, the uncertainty of its chemical composition from day to day precluded its use as a standard. The comparison of the four solutions as carriers of the bean seed extract showed the standard nutrient, i.e. 0.02 M three-salt solution, satisfactory.

TABLE VIII

EFFECT OF BEAN SEED EXTRACT IN DIFFERENT MEDIA ON THE GROWTH OF WHEAT ROOTS IN SOLUTION

Medium	Growth of wheat roots with 0, 8, and 32 ml. bean extract/1025 ml.				
	0	8		32	
	Per cent nutrient control	Per cent own control	Per cent nutrient control	Per cent own control	Per cent nutrient control
Distilled water	49	65	32	42	21
Tap water	108	41	44	23	25
1/16 nutrient	80	62	49	33	26
Nutrient	100	50	50	14	14

Other non-dormant seeds. Limited tests were made using water extracts of wheat, barley, soybean, eggplant, and tobacco. All of these were found to inhibit the growth of wheat roots when tested according to the technique described above.

Mosheov (13) has demonstrated the inhibitive influence of extracts of wheat seeds soaked in water for one to two hours on the germination of the same seeds. The action of this substance was stronger in the light than in the dark and was destroyed on extended boiling. After three to four days water extracts contained a substance, probably a nutrient, which stimulated plant growth. Smith (16) claims that the presence of chaff in varieties of wheat, barley, and oats inhibits germination and reduces mold growth in Petri dish cultures. Aqueous extracts of chaff were not consistently effective, however, nor was there any inhibition in soil plantings. Physical and chemical extracts of oats, barley, spinach, and celery have been found to have a retarding effect on beet and lettuce seed germination, but with the exception of celery, this effect was small (18).

Some work has been done to indicate that at least one of the actions of inhibiting substances is on digestive enzymes. Mature wheat endosperm has been reported to contain a water-soluble substance inhibitory to the action of salivary amylase on native wheat starch granules (10, 11). The inhibitor is absent in immature wheat kernels and appears at about the time the seed attains its mature shape. It was found in all samples of wheat and rye tested and one of similar properties in certain of the sorghums. Amylase inhibitors could not be detected in barley, oats, maize, rice, or most sorghums. According to Bowman (2, 3) aqueous extracts of ground navy beans or soybeans contain a fraction which retards the activity of pancreatic amylase and also inhibits the digestion of milk casein by trypsin. He thinks its presence may account for the low nutritive value of soy and navy beans.

A factor causing growth retardation in chicks can be extracted from unheated soybeans with dilute acid (7). This factor has properties similar to the proteolytic inhibitor and may be identical. A growth inhibitor to rats has also been found in raw soybeans (9) but it was not demonstrated to be the trypsin inhibitor.

SUMMARY

Extracts were made of dormant and non-dormant seeds and their effects on the growth of wheat roots in solution were tested. An adaptation of wheat root growth technique to this problem is described.

Seeds of *Sorbus aucuparia* and *Berberis Thunbergii*, representing a type of dormancy broken by a long or short period, respectively, in a moist medium at low temperature, were used. Extracts of these seeds made after different periods of moist storage at 5° C. inhibited the growth of wheat roots in solution, but there was no definite relation between the degree of inhibition and the progress of after-ripening of the seeds. However, the extract of *Sorbus* seeds stored for eight weeks or longer in moist granulated peat moss at 5° C. appeared less toxic to wheat roots than extract of dry seeds.

Seeds of three varieties of *Lactuca sativa* were extracted when freshly harvested and after dry storage in the laboratory for 1, 2, 4, 8, 11, and 19 weeks, and one year. No significant differences in the injurious effects of these extracts upon wheat root growth in solutions containing them were evident up to eight weeks of dry storage. Tests made after longer periods of dry storage indicated a trend toward a decreased activity of the inhibitor. This paralleled the decreasing dormancy of Grand Rapids and Iceberg varieties as shown by the ability of the former to germinate at 25° C. and the latter at 30° C., but was not related to the germination requirements of the more dormant Big Boston variety. Treatment of the extracts at 5° C. or with solutions of thiourea, both of which are effective for bringing about the germination of dormant lettuce seeds, failed to affect the inhibitor in the first instance, but indicated a possible deactivation of it in the second case.

Extracts of non-dormant seeds of *Phaseolus* sp., *Triticum* sp., *Hordeum* sp., *Glycine* sp., *Solanum melongena*, and *Nicotiana tabacum* also inhibited the growth of wheat roots in solution.

These results have led to the conclusion that dormancy is not imposed, solely, by the presence of a substance soluble in water and deleterious to the growth of wheat roots in solution. There may, however, be other types of substances, such as those which might be removed by long leaching, causing failure to germinate.

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STUDIES ON pH VALUES OF SAWDUSTS AND SOIL—SAWDUST MIXTURES

M. M. McCool

Renewed interest has arisen with respect to the value of sawdust as a mulch, for altering soil structure, and also as a fertilizer. This report deals mainly with the results of investigations on the pH values of sawdust and mixtures of sawdust and soil. The pH values of sawdusts taken from different species of trees varied, and in addition the values were raised by subjecting the sawdusts to leaching with distilled water.

LITERATURE

Turk (2) investigated the effect of mixed sawdust from hard and soft woods on plant growth under greenhouse conditions. His results induced him to conclude that the reduction in plant growth by such materials is due to a deficiency of nitrogen in the soil. "If sawdust is supplemented with some readily available source of nitrogen, it can be added to soils without harmful effects" (2, p. 21). He considers it to have no fertilizer value and its benefits are considered to be physical in nature.

Johnson (1) found sawdust mixed with soil to depress the nitrate content. When employed as a much the effect in this respect was less the first year than it was two, three, or four years after its application. He considers that old pine sawdust can be mixed with the soil without injurious effects provided a sufficient amount of nitrogen is added.

Viljoen and Fred (3) also conclude that the unfavorable action of wood on plant growth is due to lack of nitrogen in the soil.

Citations on the pH relationships of sawdusts and soil and their effects on plant growth were not located.

METHODS

The sawdust from blue beech, larch, locust, spruce, white birch, elm, hemlock, white oak, and red oak were obtained from the nursery from trees about 20 years old. The remainder came from lumber in the carpenter shop of the Institute. The fresh sawdust was placed in containers, taken to the laboratory, and pH values determined immediately.

The mixtures of sand soil, hereinafter referred to as sand, soil, and sawdust and Gloucester loam and sawdust were kept in a ventilated incubator, the temperature of which was maintained at 30° C.

The pH values were determined by means of a Beckman pH meter. One part by weight of soil and two and one-half parts of distilled water

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were mixed and the readings taken after ten minutes. One part of sawdust and 14 parts of water were mixed and let stand ten minutes with stirrings before the readings were taken. The pH values of the soils and sawdust mixtures were determined in the same manner except that one part of the mixtures and seven parts of water were employed. When the bulk samples of sawdust were thoroughly mixed before sampling only slight differences in the values, 0.1 pH value or less, were observed between replicate determinations. When the effect of leaching was studied, the sawdusts were leached slowly with distilled water by placing them in Buchner funnels.

RESULTS

The reaction or pH values of the materials studied comprise Table I. The values given are determinations on three different samples in each case. An examination of these data brings out that six of the nineteen specimens had pH values less than 5, ten between 5 and 6, and three with values of 6 or more.

TABLE I

pH OF SAWDUST FROM DIFFERENT SOURCES

Species	pH		
Yellow pine (*Pinus echinata* Mill.)	4.8	4.7	4.9
Blue beech (*Carpinus caroliniana* Walt.)	5.9	5.8	5.9
Larch (*Larix dahurica* L.)	6.1	5.0	5.1
Locust (*Robinia pseudoacacia* L.)	6.3	6.2	6.4
Spruce (*Picea glauca* L.)	4.4	4.4	4.3
White oak (*Quercus alba* L.)	4.8	4.7	4.8
Red oak (*Quercus rubia* L.)	5.6	5.0	5.1
Black oak (*Quercus velutina* Lam.)	4.9	4.6	4.8
White birch (*Betula alba* L.)	5.7	5.6	5.7
Elm (*Ulmus americana* L.)	6.2	6.1	6.2
Hemlock (*Tsuga canadensis* L.)	6.8	6.5	6.4
Sugar pine (*Pinus resinosa* Ait.)	5.2	5.3	5.2
Redwood (*Sequoia sempervirens* L.)	4.5	4.4	4.3
Maple (*Acer saccharum* L.)	5.1	5.2	5.0
Cypress (*Taxodium distichum* L.)	3.5	3.9	3.9
Mixed sawdust (unidentified)*	5.1	5.1	5.3
Pin oak (*Quercus palustris* L.)	5.7	5.5	5.8
Japanese larch (*Larix leptolepis* Gard.)	5.3	5.4	5.3
Red pine (*Pinus resinosa* Cheyney)	5.4	5.4	5.1

* From commercial source.

As the data which comprise Table II show, ten of the sawdust samples became somewhat less acid upon leaching with eight inches of distilled water.

The results derived from mixing equal volumes of sawdust and soil comprise Table III. The water content of the sand and sawdust mixtures was brought to 22 per cent and those which contained Gloucester loam to 25 per cent and were so retained throughout the test period by the addition of distilled water. It is to be noted that after 14 days the pH values of sand

and sawdust mixtures were lower in six and higher in five than was that of the sand alone. The mixtures which contained Gloucester loam soil had higher pH values than the soil alone. After 360 days four of the mixtures which contained sand soil gave lower pH values and four higher than did the soil. Thus it may be that the pH values which sometimes result from the addition of sawdust to soils may be a factor to be considered. It would appear, if the plants selected are known to be sensitive to soil reaction, that the effect of the sawdusts on the pH values of the soil should be ascertained before they are so utilized.

TABLE II

Effect of Leaching on the pH Values of Sawdust.
Average of Duplicate Determinations

Material	pH	
	Untreated	Leached with 8 inches distilled water
Sugar pine	5·2	5·6
Redwood	4·5	5·2
Spruce	4·3	5·2
Mixed sawdust*	5·1	5·5
Mixed pine shavings†	4·6	5·4
White oak	4·8	6·3
Black oak	4·9	5·3
Red oak	5·6	5·9
Maple	5·1	5·7
Pin oak	5·6	5·9

* From commercial source.
† From Institute carpenter shop.

TABLE III

pH Values of Sawdust and Soil or Sand Mixtures.
Equal Parts of Each

Material	14 days	360 days
Mixed pine and sand	5·8	5·4
Yellow pine and sand	5·9	6·2
Yellow pine and Gloucester	5·1	5·6
Mixed pine and Gloucester	5·0	5·1
Birch and sand	5·1	4·9
White oak and sand	6·0	6·2
Beech and sand	4·8	4·8
Elm and sand	6·9	6·5
Locust and sand	6·5	5·8
Hemlock and Gloucester	6·0	6·1
Hemlock and sand	5·9	6·8
Sand	5·8	5·9
Gloucester	4·3	4·4
Spruce and sand	4·8	Not run
Sugar pine and sand	5·7	Not run
Maple and sand	5·6	Not run

The effect of shavings and sawdust on the rate of percolation of water through Grundy silt loam was also investigated. The materials mixed with the soil represented one-fiftieth of its weight. The data which make up Table IV show the superiority of the mixed pine shavings over the finer or mixed pine sawdust with respect to the increase in the rate of percolation. Such relationship might not hold over a longer period or when the texture of the materials is affected by decomposition or otherwise.

TABLE IV

EFFECT OF SAWDUST ON THE RATE OF PERCOLATION OF WATER THROUGH GRUNDY SILT LOAM SOIL IN 6 HOURS

Cultural treatment	Amount of percolate in cc.		Av., cc.
Control soil, no treatment	410	400	405
Soil+mixed pine shavings	620	650	635
Soil+mixed pine sawdust	320	340	330

SUMMARY AND CONCLUSIONS

The pH values of sawdust from yellow pine, red pine, blue beech, larch, locust, spruce, white oak, red oak, black oak, pin oak, white birch, elm, hemlock, sugar pine, redwood, maple, Japanese larch, and cypress were ascertained. The lowest value was that of cypress, and the highest was that from hemlock.

The values of sawdust from sugar pine, redwood, spruce, white oak, black oak, red oak, pin oak, maple, a mixture composed of unidentified woods, and mixed pine shavings became higher upon leaching with distilled water.

The pH values of mixtures of sawdusts and soil in some cases became less and in others greater than the soils employed. Thus the acidity as measured by the pH of the mixtures of soil and sawdust may be a factor to consider in accounting for unfavorable results which may follow the additions of sawdust to soils.

Coarse material such as shavings increased the rate of percolation of water through Grundy silt loam to a greater extent than did the same material when in a more finely divided state as in sawdust.

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STOMATAL BEHAVIOR AS INFLUENCED BY TREATMENT WITH β-NAPHTHOXYACETIC ACID[1]

MARIO G. FERRI AND AUREA LEX

INTRODUCTION

Not many observations are found in the literature about the influence of hormone-like substances on the behavior of the stomates.

Boysen Jensen (1) reported the results of experiments performed with leaves of *Sinapis* and *Sambucus*. The leaves were severed and dipped in either water or growth substance solutions of several concentrations. Although considerable amounts of solution were taken up by the leaves, no effect could be detected on the degree of stomatal openings. It might be inferred, therefore, that the stomata continued to function in a normal way.

Brown (2) studied the influence of treatment with 2,4-dichlorophenoxyacetic acid (2,4-D) on the water relations of bean plants. He established a difference in the amounts of water lost by treated plants in relation to untreated controls. Control plants kept a constant rate of water loss for a period of seven days whereas the plants sprayed with a 0.1 per cent 2,4-D solution transpired at a gradually decreasing rate: during the first 23 hours the treated plants lost 21 per cent less water than did the controls; at the end of seven days the accumulated water losses were 45 per cent less in the treated plants. Brown does not report any observations on the stomatal openings.

Williams (10) has recently studied the influence of heat-shocks on stomatal movement. He suggested that the partial stomatal closure caused by the application of heat-stimuli to the leaf might be explained by the transmission of a toxin or wound-hormone from the burnt region. In the absence of further evidence it is not clear whether his observations fall into the same category of phenomena to be described here.

The present paper shows that hormone-like substances cause a stomatal closure that can be seen either indirectly through a reduction of the transpiration rate or directly by observing the stomatal openings.

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[1] The experiments reported in the present paper were completed in the laboratories of the Dept. of Botany, Faculdade de Filosofia, Ciências e Letras, Universidade de São Paulo, Brazil. The problem was suggested by Dr. P. W. Zimmerman in 1945, when the senior author was working at the Boyce Thompson Institute under a fellowship granted by The Rockefeller Foundation. It was not possible then to complete that investigation because certain equipment was needed that could not be obtained due to war restrictions. Thanks are due Dr. P. W. Zimmerman for his valuable suggestions.

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MATERIAL AND METHODS

The present experiments were performed with plants of *Tropaeolum majus* L. in four-inch pots.

Unless otherwise indicated, treatment was made by pouring into each pot 75 cc. of an aqueous solution of β-naphthoxyacetic acid at a concentration of 300 mg./l. Control plants received at the same time an equal volume of water.

The stomatal openings were observed directly under a microscope with vertical illumination or studied by the infiltration method designed by Molisch (6) and examined critically by Schorn (9). Indirect observations on the degree of opening of the stomates were made by determining the rates of transpiration. Though indirect, this is the most precise method as shown by investigations of Pisek and Berger (7), Rawitscher and Ferri (8), and others. This method is based on determinations made by weighing rapidly on a small balance (torsionswage).

A leaf severed from the plant and placed on the balance loses water and thus reduces its weight. If the first readings are made rapidly enough the stomates do not have time to modify the degree of their opening and thus the first values determined are very close to the values of water lost by the leaf still on the plant. As time goes on, since the water lost cannot be replaced there is a stomatal movement of closure that is reflected by a decrease in the transpiration rate.

EXPERIMENTAL RESULTS

Leaves cut off from untreated *Tropaeolum* plants at times when the surrounding conditions allowed the stomates to be open showed initially high values of water loss. Within a few minutes the stomates started closing and the transpiration rate fell quickly to almost constant values; at this point the stomates were closed, or almost so, and the transpiration was only cuticular (Fig. 1).

Leaves severed from treated plants (about 24 hours after treatment) showed, from the beginning, very low rates of transpiration even when the water loss was very high in the control plants. This means that in the untreated plants the degree of stomatal opening was much greater. However, the stomatal closure was not complete in the treated plants for a small decrease in the transpiration rate could still be observed there (Fig. 1).

Infiltration of the leaves with xylol and petroleum ether indicated also a smaller degree of opening of the stomates of treated plants. Direct observations of the stomatal openings under the microscope with vertical illumination also showed smaller openings in the treated plants.

When such observations and measurements were made during the successive hours of the day the daily course of transpiration for both treated

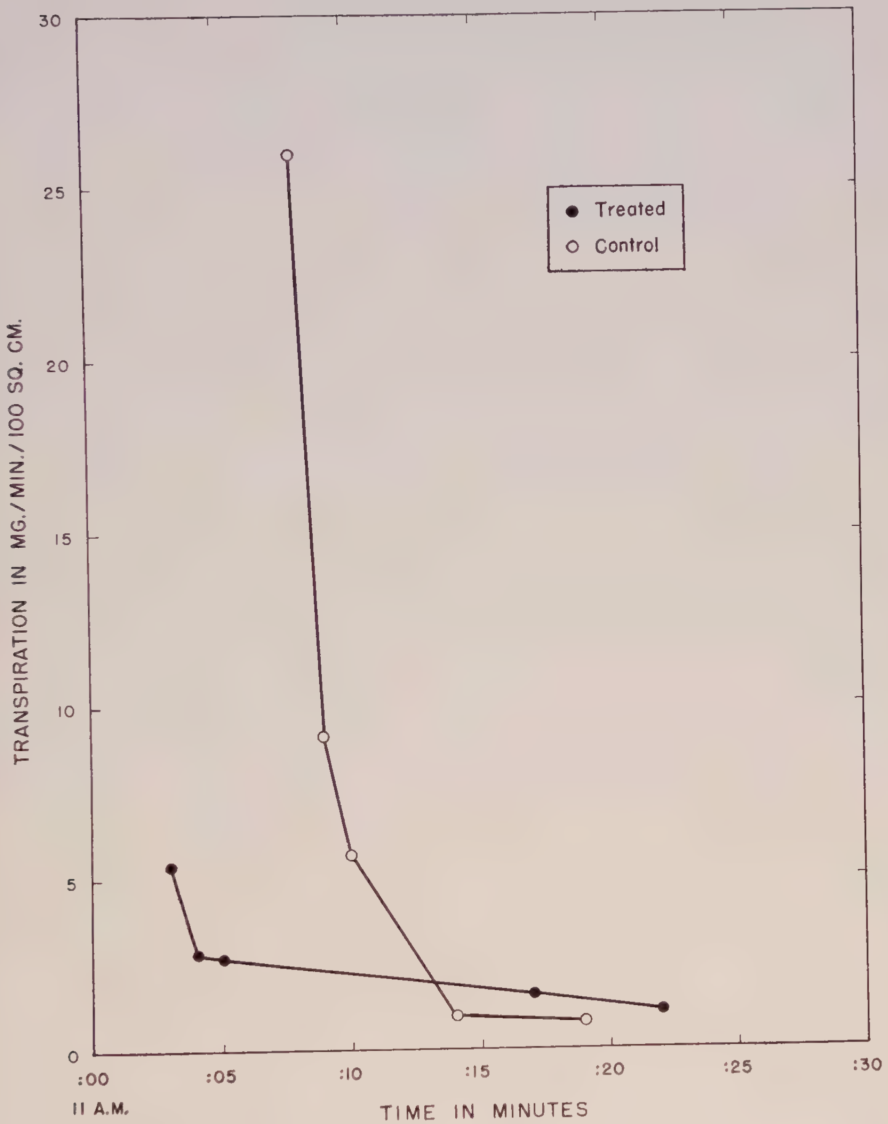

FIGURE 1. Transpiration curves of leaves severed from treated and control *Tropaeolum* plants.

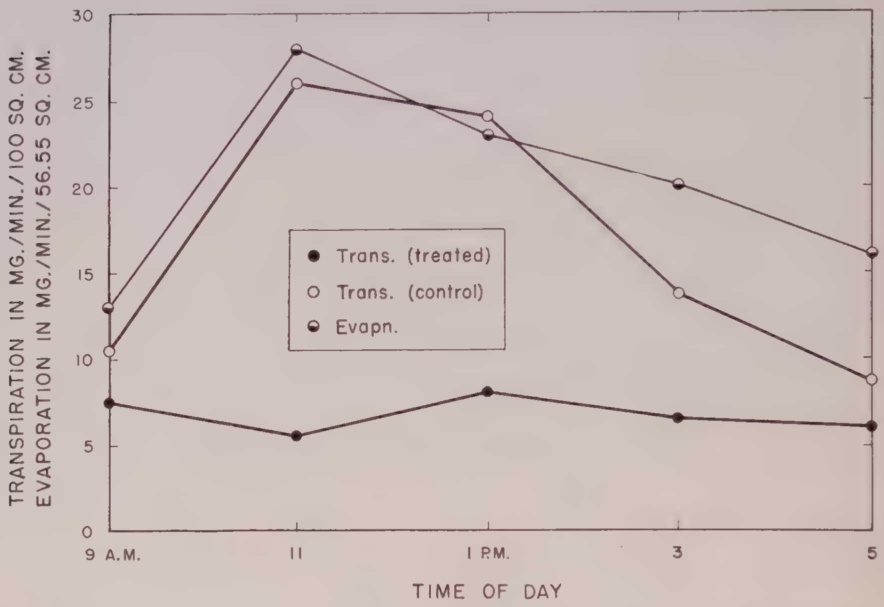

FIGURE 2. Daily course of transpiration of treated and control *Tropaeolum* plants, and evaporation rates as determined by the difference in weight caused by the loss of water from a wet disc of green blotting paper.

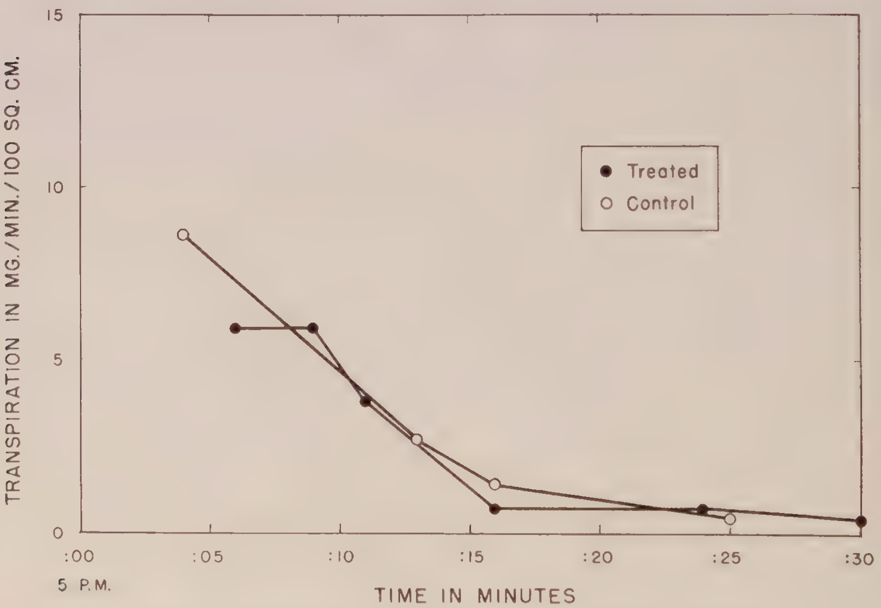

FIGURE 3. Transpiration curves of leaves severed from treated and control *Tropaeolum* plants.

and control plants was established (Fig. 2). Such a curve for treated plants showed almost constant values throughout the day whereas for the control plants the variation in the values was very great.

As the stomates of treated plants are kept closed, throughout the day, the transpiration depends more upon the surrounding conditions than upon any other factor. In the untreated plants, since the stomates retain their capacity of movement, the transpiration is influenced also by the degree of stomatal opening. Since the transpiration rate of treated plants does not greatly change throughout the day, it is evident that differences of transpiration rates between treated and control plants will depend much more on the degree of stomatal opening of the latter plants. This difference is small when the stomates of control plants are completely or partially closed (Fig. 3); it is great if they are fully open (Fig. 1). Thus it is not easy to establish with precision the degree of reduction induced by treatment on the stomatal opening.

TABLE I

EFFECT OF TREATMENTS ON TRANSPIRATION RATE

Treatment	No. of experiments	No. of times transpiration rates were the same for treated and control plants	No. of times transpiration rate was greater for	
			Treated	Controls
Soil (roots treated)	24	1	0	23
Aerial parts treated	26	8	0	18
Total	50	9	0	41

That the treatment has really brought about a reduction of stomatal opening cannot be doubted since this effect has consistently appeared throughout the experiments. In 23 out of 24 cases the transpiration rate of treated plants was lower (smaller degree of stomatal opening) and only once was it equal to that of control plants.

A few preliminary observations were performed using different methods of treatment to exclude the possibility that the activity of the roots might be affected when the solution was poured on the soil. The leaves of a few plants were sprayed with a 300 mg./l. aqueous solution of β-naphthoxyacetic acid. In other instances lanolin preparations (10 mg./g.) of the substance were applied around the petiole in a band approximately 1 cm. wide. Also a few leaves were cut off and their blades immersed in either water or a solution (300 mg./l.) for a few hours. Finally, a few experiments were performed according to the technic employed by Boysen Jensen (1), namely the petioles were cut and kept under water or the treating solution. Although the influence of treatment by any of these methods was much less evident than when the growth substance was given to the plant through the roots, the majority of the results leaves no doubt about the effectiveness of the treatment. Table I shows that out of 50 experiments the treated plants transpired less than did the controls in 41 cases and the transpiration rate was the same for both in 9 cases. Excluding the soil treatments the remaining 26 experiments show that in 18 cases the transpiration was lower in the treated plants and in 8 cases it was equal to the transpiration of the control plants. In no instance did a treated plant transpire at a higher rate than the control.

DISCUSSION

As mentioned previously, the experiments of Boysen Jensen (1) failed to show an effect of treatment on the degree of stomatal opening.

Boysen Jensen's idea was that the treatment might cause a variation in the starch content of the guard cells, and so induce a modification of the stomatal opening. Such modification was not observed; neither was any change of the starch content of the guard cells determined under the influence of treatment.

The present authors found a positive influence of treatment on stomatal behavior, but made no analyses of the starch content of the stomates.

Brown (2) had previously established a decrease in the transpiration rate of treated plants but observed no reduction in size of the stomatal openings. Brown has given much attention to the variations in the water content of the different parts of the plant. His results, however, do not show a direct correlation between decrease in transpiration rate and variation of water content in the treated plants.

During the course of the present experiments it was observed that leaves picked from treated plants wilted much more slowly than did the ones taken from controls. Also intact plants when exposed to conditions of low water supply wilted more slowly when they had been previously treated. This is due to the fact that the stomatal closure of treated plants causes a decrease in the water consumption, and so the reserves can last a longer period. This may not be advantageous to the plant, for if a partial closure of the stomates determines a decrease in water loss, it also causes a decrease of the entrance of CO_2.

Mitchell *et al.* (4, 5) have shown that bean plants treated with growth substances show an increased rate of starch hydrolysis. In contrast, Loomis (3) found that dandelion plants treated with 2,4-D contained moderate carbohydrate reserves and a high level of reducing sugars just before death of the plants occurred. According to Loomis, the loss of about half of the carbohydrate reserves by increased respiration is what would be expected from the stimulation of respiration by a protoplasmic toxin. Presumably the explanation for the action of 2,4-D or other growth substances in causing the death of bean plants by starvation cannot be used as a generalization for all species of plants. Perhaps a growth substance treatment which prevents the stomates from opening could be considered as one of the factors, in addition to the depletion of organic food reserves, in bringing about premature death of the plant.

The experiments in which the treatment was made by application of the growth substance to the aerial part, though preliminary, suggest the conclusion that the growth substance acts directly on the stomates and not indirectly through any change in the absorption or conduction power of the plant.

SUMMARY

Potted plants of *Tropaeolum majus* were treated by adding 75 cc. of an aqueous solution of β-naphthoxyacetic acid (300 mg./l.) to the soil. Control plants received 75 cc. of water. Measurements of transpiration rates, made by rapid weighings on a small balance (torsionswage), were started 24 hours after the treatment. Determinations of the stomatal openings were made at the same intervals by the infiltration method or by directly observing the stomates under a microscope with vertical illumination.

It was found that the transpiration rate changed throughout the day in the control plants, while in the treated plants the rate was almost constant. Leaves cut off from treated plants wilted more slowly than those severed from control plants. The same was true for entire potted plants with a limited water supply. Stomatal openings which fluctuated in the control plants but which were almost closed all the time in the treated plants were responsible for this behavior.

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FURTHER STUDIES ON THE OCCURRENCE OF EMBRYOLESS SEEDS AND IMMATURE EMBRYOS IN THE UMBELLIFERAE

Florence Flemion and Esther Thayer Henrickson

In the Umbelliferae the occurrence of seeds without embryos but with apparently normal endosperm is common and may reach a frequency of 50 per cent or more (7). An examination of many different lots of seeds of nine umbelliferous species (6) revealed that these embryoless seeds in many cases accounted for the great variation in the germinative capacity of fresh seeds. Further samples of seeds of some of these and of ten additional species of this family have been examined and the data are given in the present paper. The frequency of embryolessness in this group is seen to be high and, as previously observed, occurs in widely separated geographical areas.

Various factors which were thought might bring about this condition were investigated and the results of some of these studies are presented below. It had been shown previously that no significant relationship exists between the occurrence of embryoless seeds and source, variety, or crop year (6). Other possible factors such as climatic conditions, soils, genetical influence, position on the plant, etc. were investigated. As seen in the data below, none of these factors appeared to have any significant influence. However, some relationship was found between the presence or absence of embryos within a given pair of seeds. There was also a persistent tendency for seeds produced later in the season to have a higher average percentage of embryolessness.

Besides embryoless seeds, a considerable number of seeds with normal endosperm but with immature embryos frequently occurs in this family (1, 6). Many of these immature embryos are incapable of development, thus lowering the germination quality and seedling vigor. The frequency of this condition is again illustrated in the data presented below. No correlation has thus far been observed between embryolessness and the incidence of immature embryos.

METHODS AND RESULTS

Various umbelliferous seeds grown in Egypt, Norway, and in this country were examined for the presence and frequency of embryolessness by a technique previously described (7). The results with 27 samples representing 18 species (10 not previously studied) are presented in Table I. The seeds from Egypt were received July, 1947, through the courtesy of Dr. M.

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Bahgat of the Ministry of Agriculture at Giza, Egypt. Those from Norway were received November, 1947, through the courtesy of Miss Astri Frisak of the Seed Control Station at Vollebekk, Norway. The scientific names as listed in Table I are those which appeared on the seed labels.

TABLE I

OCCURRENCE OF EMBRYOLESS SEEDS AND OF IMMATURE EMBRYOS IN VARIOUS SPECIES OF THE UMBELLIFERAE

Species	Origin of seed	Cutting test, per cent*				
		Embryoless	Empty	Deteriorated	Seeds with embryo	
					Mature	Immature
Aegopodium Podagraria	Norway	15	2	0	0	83
Ammi Visnaga	Egypt	3	0	0	84	13
Angelica Archangelica L.	New York	5	0	2	89	4
Anethum graveolens	Egypt	1	0	0	80	19
“ “	Norway	1	1	0	93	5
“ “ L.	Indiana	10	0	0	90	0
Anthrescus sylvestris	Norway	10	2	3	64	21
Apium graveolens	Egypt	2	0	0	98	0
Carum Carvi	“	4	0	2	81	13
“ “ Lot #1	Norway	0	1	1	96	2
“ “ “ #2	“	8	0	2	84	6
“ *copticum*	Egypt	1	0	0	86	13
“ *Petroselinum*	“	5	0	2	76	17
Coriandrum sativum	“	0	0	2	97	1
Daucus carota	“	2	0	4	87	7
Daucus carota L. (wild carrot)	New York	0	0	0	100	0
“ “ “ “ “	“	10	0	0	90	0
Foeniculum vulgare Dulc.	Egypt	0	0	0	96	4
“ “ M.	“	1	0	1	98	0
Heracleum sp.	Norway	5	2	2	83	8
Levisticum officinale	“	1	0	0	78	21
Pastinaca sativa	“	1	0	1	54	44
Petroselinum hortense var. *crispum*	“	13	1	1	50	35
Petroselinum hortense var. *tuberosum*	“	13	0	0	37	50
Pimpinella Anisum	Egypt	13	0	5	72	10
“ *Saxifraga*	Norway	5	15	1	53	26
Myrrhis odorata Scop.	New York	0	0	0	98	2

* Averages of duplicate lots of 50 seeds each.

As seen from the data in Table I embryoless seeds appear quite frequently and in widely separated geographical areas. This is in agreement with previously published data (6). In the series shown in Table I, 15 per cent in *Aegopodium* was the highest percentage of embryolessness observed. However, as high as 62 per cent for dill (6) and 87 per cent for carrot (11) have been recorded in previous work.

In the spring of 1941, dill (*Anethum graveolens* L.) seeds of the 1939 crop containing approximately 11 per cent embryolessness were planted outside in eight random plots of 135 square feet each. The rows were three feet apart while the spacing of plants within the rows of the various plots ranged from 3 to 22-1/2 inches. The results (averages of duplicate plots) are presented in Table II. No correlation was found in these data between spacing, seed size, or yield with respect to the occurrence of embryolessness.

Although only one primary umbel is produced by each plant, it is the first and almost invariably the largest umbel to develop and as a result usually produces quite a large proportion of the total seed yield per plant as well as of a given seed crop. As seen in Table II, column 13, over 40 per cent of the seeds were obtained from the primary umbels. There was a tremendous variation in the number of seeds produced per plant, since the number of secondary and tertiary umbels (9) varied. The number of seeds produced by a given type of umbel also varied as shown for the primary umbels in Table III, column 7. Usually umbel size, as well as the number of seeds per umbel, and frequently seed size, decrease as umbels of the various types are produced on a given plant.

TABLE II

EFFECT OF SPACING OF DILL PLANTS UPON SUBSEQUENT SEED YIELD, SEED SIZE,* AND PERCENTAGE** OF EMBRYOLESS SEEDS

Plants		Primary umbels			All other umbels				Total seed yield			
Spacing, inches	No.	Yield, large seeds, %	% Embryoless: Large seeds	% Embryoless: Small seeds	No.	Yield, large seeds, %	% Embryoless: Large seeds	% Embryoless: Small seeds	Total, g.	Av. per plant, g.	% Embryoless	Primary umbel yield, % of total
22–1/2	64	100	8	—	473	81	8	8	311	4.9	8	40
12	120	100	15	—	697	85	13	9	421	3.5	12	42
6	240	99.6	9	10	1460	85	11	14	1132	4.7	11	41
3	480	99.6	10	12	1757	86	19	10	1609	3.4	13	47

* Large seeds were larger than the opening of a #16 sieve—1190 microns, while small seeds passed through a #16 sieve but failed to pass through a #20 sieve with openings of 840 microns.

** Averages of 4 lots of 100 seeds each.

In an experiment to determine whether the occurrence of embryolessness was due to some environmental factor or to a genetical influence, two different lots of dill seeds of the 1941 crop were used. These seeds had been produced in Oregon and California and contained 8 and 38 per cent embryolessness respectively. In May, 1942, plantings were made outside of both seeds and plants in each of two plots which were separated by a distance of at least a mile. In a given plot when plants which had been placed outside had finished flowering and their seeds were about mature, the plants from the sown seeds of the other lot began to flower. Thus, there was no possibility of cross-pollination of the two lots within a plot and probably none between the plots. As seen in Table III no correlation in regard to percentage embryolessness was obtained between the percentage present in the seeds planted and that obtained in the subsequent crop of seeds. In other experiments and in observations made by various seed growers, no indication of a genetical effect on the occurrence of embryolessness has been noted.

Dill has been grown by seed growers in various regions of the United States including areas having high and low rainfall as well as in irrigated areas. Soils ranging from light sandy loam to heavy adobe were utilized. There was also quite a range in temperature, for some areas fluctuated from a high of 90° F. or above during the day to cool nights of 40° to 50° F. while in other areas the temperature was less fluctuating and more moderate. The behavior of dill grown under these various conditions has been far from satisfactory for in a given locality great fluctuations in the amount of embryoless seeds occurred from year to year.

TABLE III

EFFECT OF PER CENT EMBRYOLESSNESS IN DILL SEED PLANTED UPON PER CENT EMBRYOLESSNESS OBTAINED IN THE SUBSEQUENT SEED CROP

Plantings, out-of-doors				Primary umbels					
Plot*	May, 1942	Seeds used		No.	Harvest period	Seed yield, No.		% Embryoless**	
		Source	% Embryoless			Range	Av.	Range	Av.
I	Plants†	Oregon	8	57	July 13–Aug. 7	162–1,663	819	0–64	8
	Seeds	California	38	22	Aug. 4–Sept. 21	416–3,475	1,635	0–81	17
II	Plants†	“	38	47	July 13–30	223–2,029	1,197	0–48	9
	Seeds	Oregon	8	11	Sept. 4–24	52– 905	366	1–44	20

* Spacing 15 inches apart within rows 3 feet apart.

** Averages of duplicate lots of 100 seeds each.

† Seeds planted earlier in greenhouse.

Various types of fertilizers were added to soils at Yonkers, New York, and this also had no apparent effect upon the subsequent occurrence of embryolessness in dill. Also, at Yonkers, detailed records were kept in 1945 of the various climatic conditions such as temperature, humidity, etc. preceding, during, and subsequent to the period of fertilization up to seed maturity. Here again no correlation between climatic factors and occurrence of embryoless seeds was detected. Also none of the umbels regardless of type, date of flowering, or seed maturity gave any clue as to the cause of embryolessness.

The presence or absence of embryoless seeds in the primary umbel did not in any way determine the proportion of embryoless seeds produced in the other umbels on the same plant or in any of the umbels of adjacent plants. Neither did the positions of the umbels on the plant, the umbellets within an umbel nor the florets within the umbellets reveal any correlation. However, an examination of 66 pairs of seeds of various sources revealed that in approximately 90 per cent of the cases both seeds within a pair were similar, that is, either both were with or without embryos.

Various attempts at hand-pollination were made but none was very successful in obtaining a satisfactory seed set. As flies had been found by Borthwick and Emsweller (2) to be good pollinators for carrot they were used for dill. The excellent seed yields obtained by this procedure are illustrated by an experiment conducted in the greenhouse in June, 1941. Nine dill plants caged insect-free produced an average of 59 seeds per primary umbel while nine dill plants caged with houseflies (*Musca domestica* L.) produced an average of 1,001 seeds per primary umbel. In order to avoid the necessity of adding new flies every few days, food was maintained in each cage by keeping cotton in a Petri dish moist with a 10 per cent sucrose solution. Under these circumstances the flies remained alive and active for several weeks. That some pollination occurred without insects was not surprising in view of the flowering habits of dill. Even under winter greenhouse conditions seeds were obtained when houseflies were used as pollinators; however, embryolessness under these conditions was not obtained although temperature and other factors were varied. In the summertime seeds produced in an open greenhouse occasionally contained a few embryoless seeds. While within a given set of plants grown outside the per cent of embryolessness was often very great (Table III, column 9), the average percentage tended to be greater for seeds which were produced later in the season (Table III, columns 6 and 10). In subsequent experiments this tendency persisted and was not correlated with seed size or yield.

Besides embryoless seeds in the Umbelliferae many lots contain immature embryos. That this condition is not only quite prevalent but frequently appears in high percentages is seen in Table I. Seeds with immature embryos contain firm white endosperm while the embryos may be in any stage of development (5, Tables III and IV). The mature dill embryos shown in a previous paper (7, Fig. 1 A and C), are firm and white and illustrate size variations. In experiments conducted in 1945 it was observed that seeds produced late in the season tended to have a higher proportion of seeds with immature embryos. An analysis of the data so far obtained reveals no relationship between the occurrence of embryolessness in a given lot with the incidence of seeds with immature embryos.

DISCUSSION

The erratic germination of many seed lots of some of the commercially important members of this family has long been recognized as evidenced by the rather low Federal minimum germination requirements—55 per cent for carrot and celery and 60 per cent for parsley and parsnip (8). In the past, the firm ungerminated seeds at the end of a germination test have been erroneously considered by some as hard seeds. It has been shown that embryoless seeds and seeds with immature embryos which fail to develop account for much of the low germination results obtained with fresh umbelliferous seeds (6).

Great quantities of various umbelliferous seeds are produced annually. For instance, the average yearly production of carrot seed in the United States from 1939 to 1943 was approximately 1,755,000 lbs. (10). An examination of 84 lots of carrot seeds (3) revealed that the approximate average of embryolessness was 19 per cent. In another estimate based upon the results obtained with 200 lots of various umbelliferous seeds (6), the approximate average of embryolessness found in celery and parsnip was 8 per cent, carrot 16 per cent, parsley 20 per cent, dill 24 per cent, and fennel 34 per cent. In addition, approximately 4 per cent of the carrot and parsley seeds and 14 per cent of the celery seeds contained immature embryos. Thus, in these various umbelliferous seeds a tremendous loss is incurred yearly through the handling, storage, and planting of such worthless seed.

In no way can seed size, weight, or appearance assist in segregating these seeds for both embryoless seeds and seeds with immature embryos appear normal externally and are found in seeds of all sizes in a given lot. A better method of eliminating such seeds would be the control of the contributing factor or factors.

In the summer of 1947 a survey was made of the various types of insects which visited carrot, dill, and fennel growing outside. Among these, *Lygus* bugs were observed. In the summer of 1948 extensive experiments were conducted on the effect of feeding by *Lygus* bugs on the production of embryoless seeds. The results indicate that these insects may be largely responsible for embryolessness in this family. A brief resume of these studies will be found elsewhere (4) while details of the experiments are given in the accompanying paper (5). Many of the observations and results discussed above are in accord with the findings regarding the feeding of *Lygus* bugs.

Whether the occurrence of immature embryos is due to harvesting techniques, climatic conditions, genetical influence, toxic feeding of *Lygus* bugs, or some other factor or factors remains to be determined.

SUMMARY

In further studies on the occurrence of embryoless seeds in the Umbelliferae 27 additional samples representing 18 species were examined. In agreement with previously published data, embryolessness appeared quite frequently, often in high percentages and in widely separated geographical areas. In various investigations regarding the occurrence and incidence of embryolessness in this family, there was a tendency for seeds which were produced out-of-doors late in the season to contain a higher average percentage of embryoless seeds. Also, the two seeds within a given pair frequently behaved similarly. Otherwise, there was no correlation with various factors such as spacing of plants, crop yield, seed size, soil types, climatic conditions, position on the plant, genetical influence, etc.

Besides embryoless seeds in this family, many lots contain immature embryos which are frequently incapable of germination. To date, no correlation has been detected between the occurrence of embryolessness and of immature embryos in a given lot of seeds.

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RELATION OF LYGUS BUGS TO EMBRYOLESS SEEDS IN DILL

Florence Flemion, Harriet Poole, and June Olson

A problem confronting seed growers has been the production of many poorly-germinating seed crops of such commercially important members of the Umbelliferae as carrot, celery, parsley, etc. In some cases as high as 50 per cent or more of a fresh crop of apparently well-filled umbelliferous seeds can not germinate because the embryo is lacking although the endosperm appears normal (14). In no way can these embryoless seeds be eliminated by external appearance, weight, or size differences.

Embryolessness in this family has been found to occur at random from year to year with no correlation in regard to source or variety (13), yield, soil types, climatic conditions, genetical influence, position on the plant, etc. (12). However, there was some indication that the seeds within a pair quite frequently behave similarly. Also, there was a tendency for a considerably higher average per cent of embryolessness to appear in seeds which matured later in the growing season.

A brief resume of the results of experiments conducted with the various kinds of insects which were found on umbelliferous plants appears elsewhere (11) while the full details of these studies are given below. Embryolessness occurred following the caging of *Lygus* bugs (*Lygus oblineatus* Say and perhaps others) with the developing seeds of dill. Preliminary experiments with carrot indicated that *Lygus* also produced embryoless seeds in this species of the Umbelliferae. The results suggest that these insects are responsible for the natural occurrence of embryolessness in these species.

METHODS AND RESULTS

In the summer of 1947 a survey was made of the various types of insects which visited carrot (*Daucus carota* L.), dill (*Anethum graveolens* L.), and fennel (*Foeniculum dulce* Mill.) growing outside at Yonkers, New York. Various bees, wasps, flies, beetles, and bugs were listed. It was especially noted that nymphs as well as adults of the tarnished plant bug (*Lygus oblineatus* Say) were found from the flowering period to the mature seed stage. This was of special interest in view of the literature pertaining to *Lygus* bug damage in seed production of alfalfa, beans, beets, cotton, etc.

Following a visit to the Institute in November, 1947, Dr. Kenneth F. Baker of the Division of Plant Pathology, University of California, at Los Angeles kindly arranged to have Mr. A. H. Holland of the University Extension Service collect *Lygus* bugs in Orange County, California. These

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insects subsequently arrived via air mail, but they did not survive our winter greenhouse conditions.

Lygus bugs did not begin to appear out-of-doors in Yonkers, New York on the growing dill and parsnip plants until late June, 1948. It was not until the latter part of July that adequate numbers for experiments were found here. However, several large shipments were obtained in early July through the courtesy of Mr. Robert Filmer and Dr. Phillip Granett of the Department of Entomology at Rutgers University. These insects were collected from sweepings in alfalfa fields near New Brunswick, New Jersey. In August, considerable numbers were collected in an alfalfa field near Freehold, New Jersey.

During the season of 1948 another survey was made of the insects on carrot, dill, fennel, and parsnip (*Pastinaca sativa* L.) growing on the Institute grounds at Yonkers, New York. *Lygus* were again present at all stages of seed development and increased considerably in number as the season progressed. Most of the *Lygus* collected here were from these plants. By mid-September very few of these umbelliferous plants were still growing but from then on goldenrod (*Solidago* spp.) and later *Chrysanthemum* spp. served as our chief source of supply of *Lygus* bugs.

The *Lygus* were identified as to genus by Dr. Albert Hartzell of this Institute and by Dr. Roy Melvin of the Westvaco Chemical Division of Food Machinery and Chemical Corporation, while the species identification was made by Dr. Reece I. Sailer of the U. S. National Museum. Practically all of the *Lygus* collected in New Jersey and Yonkers, New York were tarnished plant bugs (*Lygus oblineatus* Say) but occasionally other species were found.

Early in the summer of 1948 potted dill plants were transferred from the greenhouse to insect-free cages (Fig. 1) outside at the time the first umbel (primary) on each plant was showing signs of development. After the plants were placed in the cages the large door was locked and not opened until the seeds were collected when ripe. Since houseflies (*Musca domestica* L.) served as excellent pollinators for dill (12) they were kept continuously in all of the cages. A small door near the bottom of the cage (Fig. 1 A, arrow) enabled the worker to add insects or food (12, p. 295) when necessary. On each plant not only the primary but many subsequent secondary and tertiary umbels developed and excellent seed yields were obtained under these conditions.

Various types of insects which had been observed on umbelliferous plants were collected by Miss Ursula Hirsch, formerly of this Institute, and were placed in the cages containing plants. The insects were present at all stages of seed development, for throughout the experiment an ample supply of the specific type of insect was maintained in the respective cages. From two to six weeks after the first insects were added seed collections were made. From a given plant all of the umbels ripe on the same date were harvested collectively. The seeds from each such collection were thoroughly mixed, then samples of 50 or 100 seeds each were taken for the determination of the percentage of embryoless seeds by a technique previously described (14). The data obtained are presented in Table I. It is readily seen that a very high percentage of embryolessness resulted when *Lygus* bugs were present—average range of 33 to 85 per cent. Of the 32 collections made from the 16 control plants which had produced 92 umbels, only one sample contained embryoless seeds and that was only 1 per cent. Also, in the seeds produced in the presence of ants (Formicidae), fireflies (Lampyridae), Japanese beetles (*Popillia japonica* Newm.), lace wings (Chrysopidae), ladybird beetles (Coccinellidae), and syrphid flies (Syrphidae) only occasionally was any embryolessness observed. Whether this low but infrequent occurrence was due to the specific caged insects, to very small nymphs of *Lygus* which were able to pass through the wire or plastic mesh, to entry when the large door was opened for seed collecting, or to some other insects or causes, has not yet been determined. However, in

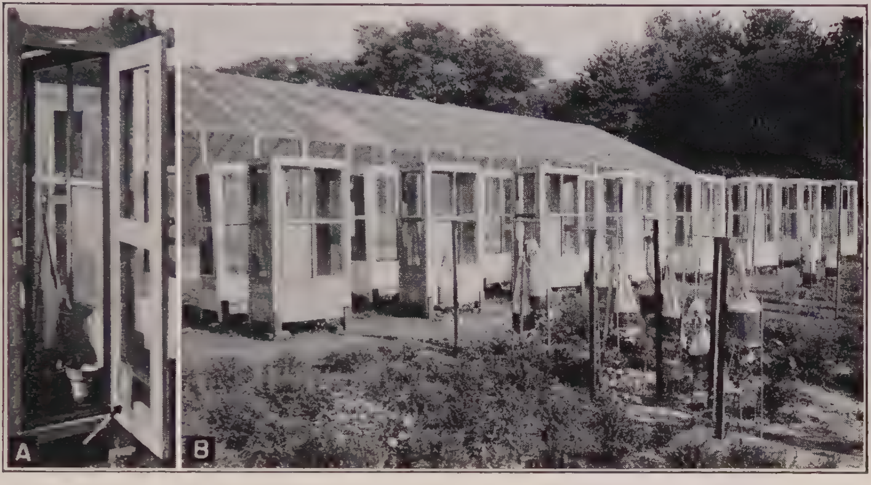

FIGURE 1. Insect-free cages. A and background of B, large cages approximately 2 feet wide, 3 feet long, and 5-½ feet high. Roof, back, and door each panelled with wire screening (34×50 mesh, 0.006 filament). The other two sides each had three panels of glass and one of lumite (plastic mesh 32×36, 0.012 filament). Wooden floor bottom covered with lumite had adequate drainage through the spacings between the boards. A, arrow indicates small door. Foreground of B, sleeve cages—cylinders 7-½ inches in diameter and 7-½ inches in height made of wire screening (24×24 mesh, 0.012 filament) with cloth fitted tightly at each end forming a sleeve. Bottom cloth gathered about stem of plant and held by fine copper wire while upper cloth fastened to overhanging wire or T-shaped stakes.

TABLE I

EFFECT OF VARIOUS INSECTS CAGED WITH DILL PLANTS UPON THE PRODUCTION OF EMBRYOLESS SEEDS

Dill plants,* caged outside							% Embryoless	
Insects added				Harvest				
Kind	Date	Cage label	No. of plants	Date	No. of umbels	No. of collections made	Av.	Range
Ants	June 30	D	3	July 19–26	18	5	0	0
Fireflies	July 6	I	6	“ 16–26	26	11	0.5	0–4
Japanese beetles	“ 16	J	6	“ 19–22	22	6	0.5	0–2
Lace wings	“ 6	C	3	“ 19–26	32	9	0	0
Ladybird beetle	June 29	A	3	“ 19–Aug. 2	42	13	0	0–1
Lygus bugs	“ 30	F	5	“ 12–26	50	20	37	0–100
	July 6	N	6	“ 19–Aug. 5	51	17	33	0–88
	“ 9	B	3	“ 23– “ 5	38	13	68	10–92
	“ 13	H	7	“ 26– “ 5	39	13	51	7–89
	“ 16	P	5	“ 26– “ 16	29	23	72	2–100
	“ 19	O	5	“ 30– “ 16	63	52	85	17–100
Syrphid flies	“ 6	K	6	“ 18– “ 4	31	13	0.7	0–3
Controls (no insects)		E	5	“ 16– “ 2	42	15	0	0–1
		L	6	“ 16–20	37	10	0	0
		M	5	“ 16–29	13	7	0	0
Outside field plantings (uncaged)			9	“ 23–29	43	10	4	0–22

* In eight-inch pots in soil.

none of these were high percentages of embryoless seeds obtained as in the case with *Lygus*.

That seeds can be kept free of embryolessness by screening the developing seeds is illustrated by the results of an experiment shown in Table II. In two lots, houseflies were placed inside the sleeve cages (Fig. 1 B) during pollination, then one of these lots remained subsequently caged free of insects while in the other lot the sleeve cages were removed and the developing seeds were left exposed to outside insects. In the third lot the plants were exposed to outside conditions (uncaged) throughout the entire period of the experiment. Regardless of the type of pollinating insects an average of 30 to 40 per cent embryolessness was obtained when no protection was afforded the developing seeds. However, when the umbels were sleeve-caged during this entire period all of the seeds produced contained embryos.

Only primary umbels were used in the experiments shown in Tables II, III, and IV. These umbels were individually harvested and representative samples of 50 or 100 seeds taken from each were examined for embryolessness.

The data in Table II illustrate, as shown previously, that the occurrence of embryolessness bears no relationship to seed yield (12). It has also been shown (12) that the average per cent (but not necessarily the range) of embryolessness tended to be greater for seeds which were produced later in the season. This fact is again illustrated by the results (Table I, last line) with seeds which matured in late July as compared with those which ripened in early September (Table II, 3rd line from bottom).

TABLE II

EFFECT OF EXCLUDING OUTSIDE INSECTS DURING THE PERIOD OF DILL SEED DEVELOPMENT UPON THE OCCURRENCE OF EMBRYOLESSNESS

Potted dill plants, outside		Primary umbels, seeds produced*		
Pollinators**	Subsequent treatment	% Embryoless	Av.	Yield, No.
Outside insects	Outside (uncaged)	24, 36, 38, 62	40	403, 240, 862, 260
Caged houseflies	" "	8, 14, 28, 68	30	505, 545, 643, 561
" "	" (caged)	0, 0, 0, 0	0	472, 510, 421, 458

* Harvested September, 1948.
** August, 1948.

Another experiment with potted dill plants was conducted in the large outside cages with houseflies as pollinators. *Lygus* bugs were added as shown in Table III, column 1, at various stages of seed development. The respective cages were then kept well supplied with these insects until seed maturity. Here again, in every case, very high percentages of embryoless seeds were obtained with *Lygus*. Three of the 14 control plants which were in very poor condition and produced only 16, 30, and 36 seeds contained 0, 7, and 8 per cent embryolessness respectively. The cause of embryolessness and of the low seed yield in these controls was not determined. However, the average percentage of embryoless seeds of the 14 controls was 1 per cent in contrast to the high percentage when *Lygus* were added even at a rather late stage of seed development.

Another similar experiment was undertaken except that the *Lygus* bugs were kept for a period of only 48 hours at various stages of seed development. Throughout this entire experiment before, during, and after the primary umbels were sleeve-caged with *Lygus* these plants were kept outside in the large insect-free cages with houseflies as pollinators. The data obtained appear in Table IV. It is seen that quite high percentages of embryoless seeds were produced by one adult *Lygus* bug when caged for only 48 hours with the primary umbels. The very young *Lygus* were also destructive, for in two cases four nymphs each were caged for 96 hours (Table IV, lines 8 and 9 from the bottom) and they, too, produced considerable damage for approximately 50 per cent of the seeds were embryoless. In this experiment the average percentage of embryoless seeds for the 14 controls was 0 per cent.

TABLE III

EFFECT OF CAGING LYGUS BUGS WITH DILL PLANTS AT VARIOUS STAGES OF SEED DEVELOPMENT UPON THE PRODUCTION OF EMBRYOLESS SEED

Stage of development when *Lygus* added	Primary umbels*				
	No. of plants	Av. No. of seeds per plant	No. of seeds examined	% Embryoless	
				Av.	Range
Umbels flowering	9	18	162	62	33–100
Stamens just fallen	5	11	53	62	0–100
Seed set, ovaries swelling	7	248	350	75	50–86
Ovaries swelling, fruit green, endosperm jelly-like, embryo not visible	7	220	350	81	66–96
Fruit full-size, styles no longer prominent, endosperm firm and white, embryo gelatin-like in very small cavity	6	265	300	80	48–96
Endosperm firm and white, embryo visible to naked eye, cotyledons not developed	5	401	250	81	62–94
Endosperm firm and white, embryo nearly or fully-developed	3	403	150	25	6–48
Control (no *Lygus*)	14	115	984	1	0–8

* Seeds collected late August and early September, 1948.

In ripe seeds which were embryoless the small embryo which lies imbedded in the endosperm at one end of the seed was lacking and instead there was a cavity. When *Lygus*-injured seeds which had been collected when ripe were examined immediately, the cavity was in some cases macroscopically devoid of an embryo while in other instances small particles of the shattered embryo were visible at the periphery of the cavity. When the embryo was only partially destroyed, it could be mistaken for an

TABLE IV

EFFECT OF CAGING PRIMARY UMBELS OF DILL PLANTS AT VARIOUS STAGES OF SEED DEVELOPMENT WITH LYGUS BUGS FOR 48 HOURS UPON THE PRODUCTION OF EMBRYOLESS SEEDS

Lygus with primary umbels for 48 hours						Controls, % embryoless
Stage of development			*Lygus** added		% Embryoless	
Fruit	Endosperm	Embryo	Date	No.		
Green and enlarging	Scarce, not firm	Not visible	Aug. 30 Sept. 1	1 2	4 2	0 0
	White and firm	Very small, jelly-like, transparent	Aug. 25 “ 23 Sept. 1	1 2 2	10 16 10	0 0 0
		Small, jelly-like, groove visible	“ 10 “ 10	4** 4**	56 46	0 1
Green, full-size	“ “ “	Cotyledons forming	Aug. 30	1	0	0
Greenish-brown, full-size	“ “ “	Small	“ 30	1	18	0
		Nearly full-size	“ 23	2	86	0
		Well-developed	“ 25 Sept. 1	1 2	18 22	0 3
		Full-size	“ 8	2	0	0
Almost brown, full-size	“ “ “	“ “	“ 8	2	72	0

* Adults.

** Nymphs for 96 hrs., all other experiments adults for 48 hrs.

immature embryo. However, if the intact seeds were kept dry in the laboratory for a few weeks and then examined, the immature embryos were still in about the same condition as when collected while the *Lygus*-injured embryos had disintegrated and in some cases the dried, shriveled tissue was no longer visible to the naked eye. Seeds were examined at various stages of development and embryolessness could be detected as soon as the endosperm was almost or completely firm regardless of the stage of embryo development; however, partially destroyed immature embryos could not be identified readily. All of the data regarding embryolessness presented in the tables were obtained by examining the seeds when ripe.

In the data regarding seed yield (Table III, column 3) there appears to be a considerable reduction in the number of seeds produced when *Lygus* were present during the flowering period. This will be investigated further, for these insects may destroy the ovary at this early stage of seed development.

In preliminary tests for the control of *Lygus* three insecticides, DDT

[1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane], nicotine, and TEPP (tetraethyl pyrophosphate) were used. For each concentration both adults and nymphs of *Lygus* were used. The solutions were sprayed directly on the insects by using a hand air-compression sprayer. In three separate tests with TEPP all of the insects were dead within 1½ hours after spraying with concentrations of 0.125, 0.06, and 0.03 per cent. The killing effect of the other two insecticides was much more variable and not as rapid. TEPP hydrolyzes so rapidly that no residual killing effects occur. In these experiments no deleterious effects on the plants have been noted to date with any of the TEPP concentrations tested.

Effective and rapid control of *Lygus* bugs is especially helpful in tests with carrot, for *Lygus* (adults as well as nymphs) hide in the tightly-closed umbels and are often not detected. In a few tests with both the wild and cultivated carrot some indication was obtained that *Lygus* bugs also produce embryoless seeds in this species of the Umbelliferae.

LITERATURE REVIEW

Lygus bugs are very widely distributed in many parts of the world. They are rather omnivorous feeders and attack a wide range of plants—more than 50 economic plants as well as many weeds and grasses (21). These insects reduce seed yields by producing bud blasting, blossom and young fruit drop, general dehabilitation of the plant, and also partial or complete destruction of the seeds. *Lygus* injury in alfalfa seeds results in the formation of shriveled or misshapen discolored seeds (5, 28, 32, 36, 37), while with the common and lima beans and also blackeye cowpeas discolored, scarred, spotted, or pitted areas are found on the seeds (2, 15, 27). In sugar beet seeds there is apparently no great reduction in the number or size of the seed balls but there is a marked decrease in the per cent of viable seeds (16, 17). In cotton, destruction of the seed by *Lygus* bugs usually occurs at an early stage of development (23, 29) and with guayule the embryo collapses when these insects feed on the seed at the pre-dough stage (26).

In some cases (27, 36) very little or no evidence of external injury resulted from the *Lygus* feeding on seeds, but internally there was considerable breakdown of the tissue. The mechanics of feeding by *Lygus* have been studied. These insects pierce the tissue with their fine, sharp, needle-like mouth parts and suck out the juices (1, 30). The actual mechanical injury is slight compared with the resulting damage, for there appears to be a toxic reaction upon the plant cells near the puncture (2, 5, 19, 20, 23, 30). Even a short feeding period causes damage (10, 32).

In New York State, the tarnished plant bugs pass the winter as adults in sheltered areas and become active the first warm days of spring (8). In the early part of the season *Lygus* bugs build up quite large populations on many of their wild and cultivated hosts. Since they reach their highest population by mid or late summer, the first pods set in lima bean (2) tend to have much lighter infestations of these insects. Population trends of *Lygus* have been studied (31, 34) and large scale migrations of adults take place from field to field (23, 33). This shift in population is determined in part by the condition of the host plant (27).

The many food plants, the migration of adults, and the almost continual hatching of nymphs make any attempt at control of these insects rather difficult, for protection must be offered to buds, blossoms, and developing seeds over long periods of time (26, 31). Various control methods have been suggested (3, 19, 29, 33, 36) and insecticides have been tested (7, 19, 22, 23, 24, 25, 35). It has been observed that nymphs sometimes differ from adults in their response to insecticides (31). That the nymphs cause damage as well as the adults (17, 26, 29, 36) is of importance since at certain seasons the nymphs are far more numerous than the adults.

There are other insects which produce feeding damage to seeds which is somewhat similar to that produced by various *Lygus* species such as: *Adelphocoris* spp. (15, 19), another member of the Miridae, and several stinkbugs of the family Pentatomidae—*Chlorochroa sayi* (16, 17, 18, 29), *Euschistus variolarius* (15), *E. impictiventris* (18), and *Thyanta custator* (16, 29).

That not all seed failures are due to insects is seen in alfalfa where other factors (4, 6, 38) may adversely affect seed development.

DISCUSSION

Many of the observations recorded in attempting to determine the cause of embryolessness in dill (12, 13) are in agreement with the feeding habits, hosts, population trends, etc. of *Lygus* bugs. This fact together with the results presented above suggest that these insects are largely responsible for the natural occurrence of embryoless seeds in dill. However, it will not be surprising if other *Lygus* species or other insects or factors can also produce embryolessness in umbelliferous seeds.

In regard to *Lygus* bugs attacking plants of the Umbelliferae, *L. campestris* feeds on various members of this family (21, p. 154), both nymphs and adults of *L. elisus* and *L. hesperus* have been found on carrot (27), and *L. oblineatus* often injures celery plants (8, p. 470) and is very abundant in the nymphal stage on wild carrot in late summer and fall (9).

For the experiments with dill and carrot described above, the tarnished plant bug (*Lygus oblineatus* Say) was used as it was the most predominant *Lygus* species in this area. This species has passed for many years in this country as *L. pratensis* but it does differ from the European *L. pratensis* (21, p. 148). In the present studies with dill plants which had been caged with *L. oblineatus*, many hatchings of nymphs subsequently occurred.

Under caged conditions when *Lygus* bugs were allowed to feed on developing dill seeds they were able to destroy the embryos at almost any stage of development but caused no apparent damage to either the endosperm or the fruit coats. Nymphs as well as adults were destructive. Some indication was obtained that these insects may reduce the seed crop when feeding occurs at or near the flowering period. Although *Lygus* bugs feed at random, an individual insect may attack both seeds of a given pair during one feeding period. Thus, pairs of seeds tend to be alike (12) in regard to the absence or presence of embryos. There was a persistent tendency for seeds produced out-of-doors later in the season to have a higher average percentage of embryolessness. This increment in incidence of embryoless seeds as the season progresses can be attributed to the population increase of *Lygus* bugs.

Prior to obtaining the above results regarding the destructive feeding of *Lygus* bugs, various possible factors such as source, crop year, variety, seed yield, climatic conditions, genetical influence, etc. were studied but no relationship of any of these with the occurrence of embryolessness was revealed (12, 13).

SUMMARY

Various insests which visited the flowers and developing fruits of various members of the Umbelliferae were caged with dill plants. Embryoless seeds almost invariably occurred and usually in very high percentages on the plants or specific umbels which had been caged with *Lygus oblineatus* Say. Even when one adult *Lygus* was caged for only 48 hours with an umbel, a tremendous amount of damage occurred to the embryos. Nymphs were also found to be very destructive. Except for a few cases, no embryoless seeds were produced in either the control plants (caged insect-free) or in plants caged with other insects. Preliminary results with carrot indicated that *Lygus* bugs can also produce embryoless seeds in this umbelliferous species. Experiments are being undertaken to determine whether other insects or factors also produce this condition or whether only *Lygus* bugs are responsible for the natural occurrence of embryoless seeds in this family.

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EFFECTS OF SOME SURFACE-ACTIVE AGENTS ON THE GROWTH OF WHEAT ROOTS IN SOLUTIONS

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INTRODUCTION

In connection with studies at this Institute on various factors influencing the germination of seeds and the growth of seedlings, the present paper reports the effects observed on the growth of young wheat seedlings of several strongly surface-active agents. These were selected from the different chemical types of the numerous commercial products which are now available and are extensively employed, more or less specifically depending on their properties, as emulsifying agents, wetting agents, detergents, disinfectants, and for other purposes.

In this study it was found that all the surface-active agents tested were effective in diminishing root growth, some more than others, and that at extremely low concentrations a few appeared to cause slight increases in root growth. The germinated seeds, which had not been previously treated with the surface-active agents, were transferred to cultures with the roots extending into nutrient solutions containing low concentrations of the test substances and allowed to grow. In this respect, especially, the test procedure was different from those used in the relatively few published studies on the effect of surface-active agents by other investigators, who usually treated the seeds with the surface-active agent prior to germination.

Zigmund (21, 22, 23, 24), in his extensive studies on the effects of a large number of chemical compounds and natural products on the germination and growth of seeds by his procedure (21, p. 300), included (22) saponin and sapogenin, both of which gave retarding effects. Traube *et al.* (17, 18) tested various substances on seeds and ascribed the effects to the influences of the substances on the surface tension. Curini-Galletti (7) reported that soaking oleaginous seeds in a 0.1 per cent saponin solution for two hours hastened germination. Balansard *et al.* studied the effects of saponins on the germination and growth of wheat (1), tomatoes (3), and corn (4), and also the effects of sodium lauryl sulfate on wheat (2). They found that presoaking the seeds in dilute solutions of these surface-active agents hastened germination and growth, but when the solutions were applied to growing seedlings or plants the effects were unfavorable. It was found by Euler (8) that seeds of *Lepidium sativum* and *Hordeum vulgare* when germinated on filter paper moistened with 0.02 to 0.05 per cent solutions of digitonin, quillaja saponin, or sodium cholate developed roots only

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a minor fraction as long as the controls, but the same length coleoptiles. Macht and Krantz (13) proposed that tincture of digitalis be assayed on the basis of its inhibition of the growth of rootlets of *Lupinus albus*.

It is seen that saponins have been the strongly surface-active agents most studied with respect to effects on seeds and seedlings. These chemically complex substances, the structures of which are incompletely known in many instances, are glycosides of sapogenins (9, p. 317–337) and occur widely in the plant kingdom. Their occurrence in various seeds (10, 15, 16, 20) is of special interest and it has been suggested (3) that they may have some natural function in the germination process. Besides saponins, other surface-active substances often present in seeds include phospholipides (phosphatides) (14, p. 365) and sterols. Interactions of these three possibly may be involved. Certain saponins, for example, digitonin, form molecular compounds with sterols (9, p. 322). In connection with the various possible functions of phospholipide, it has been pointed out (6, p. 244–252; 14, p. 376–379) that in animal tissues the phospholipide/cholesterol ratio is considered to be significant, and that these two substances are believed to have antagonistic actions in influencing certain characteristics of the cells controlling such processes as the uptake of water and salts. Perhaps a similar relationship between the phospholipides and phytosterols in seeds may be considered.

Veldstra and Havinga (19) point out that each of the following substances contains an unsaturated lactone ring in its structure: the cardiac glycosides which are medicinally important saponins in digitalis; coumarin and parasorbic acid which are inhibitors for seeds and seedlings; and certain other physiologically-active compounds. These authors consider the presence of an unsaturated lactone ring to be related to the activities of these substances. In view of this, the question may be raised as to whether the activity of a saponin should be ascribed entirely to its surface-active properties, entirely to some particular structural group, such as an unsaturated lactone ring, or partly to each.

SURFACE-ACTIVE AGENTS TESTED—THEIR CHEMICAL NATURES

The surface-active agents used (Table I) were, in most cases, commercial products some of which contain some inert material, such as, for instance, sodium sulfate. The exact chemical designations have not been completely disclosed in all instances. The designations given in Table I were obtained from a recent compilation (12) or from the supplier.

PROCEDURE

A detailed description of the procedure has been given in a previous publication (5). In brief it was as follows. Beakers containing the solutions

TABLE I

SURFACE-ACTIVE AGENTS TESTED

Type	Trade name	Supplier	Chemical designation
Non-ionic	Tween 20	Atlas Powder Co.	Polyoxyethylene sorbitan monolaurate
	Tween 85	" " "	Polyoxyethylene sorbitan trioleate
	Carbowax 400 mono-oleate	Carbide & Carbon Chemicals Corp.	Polyethylene glycol of average molecular weight of 400 esterified with one mole of oleic acid
	Saponin (purified)	Eimer & Amend	Plant source not specified
Anionic	Vatsol OT (Aerosol OT)	American Cyanamid Company	Di-(2-ethylhexyl) sodium sulfosuccinate
	Aquarex D	E. I. duPont de Nemours & Co., Inc.	Sodium lauryl sulfate
	Igepon T	General Dyestuff Corporation	$C_{17}H_{33}CON(CH_3)CH_2CH_2SO_3Na$
	Nekal BX	General Dyestuff Corporation	Isobutylnaphthalene sodium sulfonate
Cationic	Hydrocide 10-X Special	Rohm & Haas Company	A liquid concentrate of *p-ter*-octylphenoxyethoxyethyl-dimethyl-benzylammonium chloride
	Katapol CNS Conc.	General Dyestuff Corporation	A liquid concentrate of a quaternary salt having the nature of a polyethylene ether of a fatty amine
	Isonol DL1	Onyx Oil and Chemical Co.	Dilauryl-dimethylammonium bromide as a 75 per cent solution in isopropanol
Phospholipide	Astec HS	Associated Concentrates, Inc.	A water-soluble product made from soybean phosphatides as a 40 per cent solution
	Asolectin	Associated Concentrates, Inc.	A special oil-free preparation of natural soybean phosphatides

to be tested were covered with perforated, paraffined cheesecloth. Wheat[1] (*Triticum* sp.) seeds were germinated on moist filter paper in the laboratory. When the roots measured 6 to 7 mm., the seedlings were transferred to the beakers containing the test solutions in such a way that the primary roots extended through the cheesecloth perforations into the solutions, while the attached seeds remained on top of the cheesecloth. After 64 to 68 hours' growth in the dark at a constant temperature of 20° C. the primary root length of each seedling was measured. In each case, the average initial length was subtracted from the final length in the calculation of the percentage of control growth. Duplicate lots of 25 seedlings each were used for each solution.

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[1] Marquis, 1946 crop, from the North Dakota Agricultural Experiment Station, Fargo, N. D.

The control solution was 0.02 molar with respect to each of the three nutrient salts, calcium nitrate, magnesium sulfate, and potassium dihydrogen phosphate. Each test solution contained in addition the amount expressed in parts per million (p.p.m.) of a surface-active agent as indicated in Table II.

TABLE II

GROWTH OF WHEAT ROOTS IN NUTRIENT SOLUTIONS CONTAINING DIFFERENT SURFACE-ACTIVE AGENTS

Agent	Growth, as % of control, at various concns. in p.p.m.						
	1280	320	80	20	5	1.25	0.3125
Tween 20	54 54	70 66	82 83	90 95			
				104 96	104 87	91 103	
Tween 85	78 77	86 82	86 87	81 86			
Carbowax 400 mono-oleate	53 49	81 71	74 81	95 95			
Saponin		12 12	21 22	39 39	51 56		
Vatsol OT		0 0	0 1	8 8	76 77		
					77 75	94 93	94 93
Aquarex D		0 0	1 2	35 40	77 76		
					84 85	100 94	107 110
Igepon T		34 34	40 38	45 48	82 78		
					89 89	99 108	105 103
Nekal BX		1 1	16 15	74 74	119 114		
					124 122	124 120	115 116
Hydrocide 10-X Special			0 0	6 5	54 53	66 68	
						64 63	82 74
Katapol CNS Conc.			16 15	40 41	101 111	116 114	
						111 113	105 103
Isonol DL1			1 3	9 9	45 48	81 83	
						82 86	112 117
Astec HS		15 16	38 35	63 63	85 91		
					104 109	86 104	114 122
Asolectin		57		91			
				96 108	99 111	103 112	

RESULTS AND DISCUSSION

The effects on root growth are tabulated in Table II. Both of the percentage values obtained with the duplicate beakers run simultaneously are recorded. The percentage values given on the lower lines were obtained at later times when the investigation was extended to lower concentrations and it may be observed that in most cases good reproducibility was obtained in the overlapping portions of the series.

In all cases the growths of the coleoptiles (not recorded in the table) were not much different from the controls except when the root growths were reduced to very low percentages, in which cases the coleoptile growths also were reduced somewhat but not to correspondingly large degrees.

The effects of the first four substances in Table II (the non-ionic type) at 320 p.p.m. are illustrated in Figure 1 A. The control (Fig. 1 D) also will serve as the basis of comparison in the following figures. Tween 85 and Carbowax 400 mono-oleate do not form true solutions but rather dispersions, as can be observed by the turbidities in these two cases. The first three substances are relatively weakly inhibitory, but saponin is strongly inhibitory.

The effects of the next four substances of Table II (the anionic type) at 20 p.p.m. are shown in Figure 1 B. At the higher concentrations these substances are all strongly inhibitory. Throughout the range of concentrations there is no consistent parallelism in the effects of these substances. While at 320 and 80 p.p.m. Nekal BX is more inhibitory than is Igepon T, at 20 p.p.m. the order is reversed, and at 5 to 0.3125 p.p.m. Nekal BX is definitely stimulatory.

The effects of the next three substances of Table II (the cationic type) at 5 p.p.m. are shown in Figure 1 C. As seen from the table, Hydrocide 10-X Special still shows definite inhibition at a concentration of only 0.3125 p.p.m. Joslyn *et al.* (11), using the pure compound contained in this product, found it to be a very effective bactericide and fungicide. Also Isonol DL1 is still inhibitory at 1.25 p.p.m. Katapol CNS Conc. is less inhibitory at 80 and 20 p.p.m. than the other two members of this type tested, although still strongly inhibitory at these concentrations, and appears to be stimulatory at 1.25 p.p.m.

Astec HS is inhibitory at the higher concentrations but not at 5 p.p.m. (see Table II) or lower concentrations. Asolectin is probably slightly inhibitory at higher concentrations but not appreciably so at 20 p.p.m. or lower. In a previous preliminary study published from this Institute (5) it was suspected that phospholipides in an extract from pole beans may have been responsible for inhibition of wheat root growth. Assuming similarity of the phospholipides of soybeans (Asolectin) and of pole beans, this now would appear unlikely.

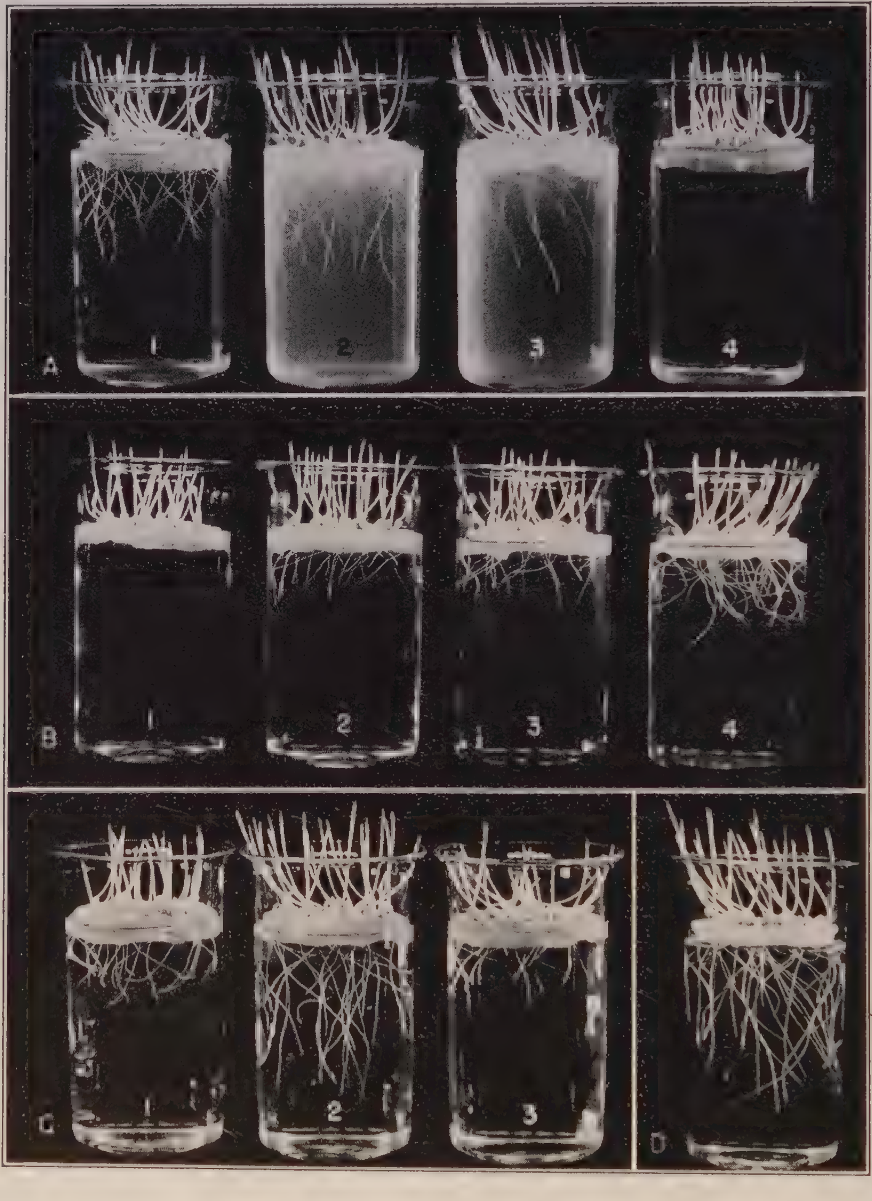

FIGURE 1. Growth of wheat roots. A. In solutions containing 320 p.p.m. of non-ionic surface-active agents: 1, Tween 20; 2, Tween 85; 3, Carbowax 400 mono-oleate; 4, saponin. B. In solutions containing 20 p.p.m. of anionic surface-active agents: 1, Vatsol OT; 2, Aquarex D; 3, Igepon T; 4, Nekal BX. C. In solutions containing 5 p.p.m. of cationic surface-active agents: 1, Hydrocide 10-X Special; 2, Katapol CNS Conc.; 3, Isonol DL1. D. In control solution.

In regard to stimulatory effects it may be stated with due consideration of the obvious experimental errors involved that the data in Table II indicate Nekal BX at 5 to 0.3125 p.p.m. and probably also Katapol CNS Conc. at 1.25 p.p.m. to be stimulatory under the conditions of these tests in view of the consistent values considerably above 100 per cent of the growth of the roots of the controls. Other isolated values above 100 per cent are hardly sufficient to indicate stimulation.

While these tests necessarily have been limited to only a few of many surface-active agents now available, it may be anticipated that others also will exhibit some similar effects, possibly in some cases even to greater degrees.

SUMMARY

Thirteen different surface-active agents selected from the various types have been tested at a series of low concentrations for their effects on the growth of wheat roots in a solution of nutrient salts.

The three commercial agents of the non-ionic type and also soybean phosphatides show little effect, inhibition being evident only at relatively high concentrations.

Saponin, a natural agent of the non-ionic type, is very inhibitory.

The four anionic and the three cationic agents, and also a water-soluble product derived from soybean phosphatides were all found definitely inhibitory at concentrations as low as 20 p.p.m., and several were found still inhibitory at lower concentrations. Two of the surface-active agents, namely, Nekal BX and Katapol CNS Conc., were found to be slightly stimulatory at very low concentrations.

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CULTURE AND USE OF ENTOMOGENOUS FUNGI FOR THE CONTROL OF INSECT PESTS[1]

Edgar Dresner[2]

This report is concerned primarily with a study of *Beauvaria bassiana* (Bals.) Vuill. It includes observations on the methods used in the laboratory production of spore material on artificial media together with the results of laboratory and field applications of *B. bassiana* dust for the purpose of determining host range and practicability of field control.

Two genera of fungi are already well known as insect parasites, *Beauvaria* and *Metarrhizium*. *B. bassiana* is listed by Charles (1) as causing the death of many species of insects. *Metarrhizium glutinosum* is not listed; it was identified in 1944 by Pope (7). *Metarrhizium anisopliae* (Metsch.) Sor. has a host list of over 40 species of insects (Charles, 1).

MATERIALS AND METHODS

In May, 1947, the American Type Culture Collection, Georgetown University, Washington, D. C., furnished the writer with stock cultures of *Beauvaria bassiana* and *Metarrhizium glutinosum* Pope. In preliminary tests spores produced by artificial culture of these two fungi applied to insects resulted in death of the insects with *B. bassiana* only. The very low spore production rate in culture on media and the lack of toxicity were the reasons for the early elimination of the culture and testing of *M. glutinosum*.

CULTURE OF BEAUVARIA BASSIANA

The use of agar slants in test tubes for the culture of this fungus while involving little danger of contamination was not considered suitable for the large scale production of spores. Test tube cultures were maintained only for the production of spores for seeding bulk cultures. Potato-dextrose agar, molasses agar, and lima bean agar were successfully used. The molasses agar was found to be the best; it produced an even, almost luxuriant spore growth in contrast to the other media which were characterized by isolated tufts and low spore production. The molasses agar was

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made up of 300 ml. of molasses, 700 ml. of distilled water, 20 g. of flaked agar agar, and 1 g. of sodium chloride.

Large scale production of *B. bassiana* spores was accomplished by cultures on vegetable media. Bulk media successfully used for large scale production included:

Bran (ten parts by weight of bran, one part of water)
Lima beans (*Phaseolus limensis* Macf.), whole, water-saturated
Tendergreen bush beans (*Phaseolus vulgaris* L.), whole, water-saturated
Tendergreen bush beans, water-saturated and ground to a paste
Corn (*Zea mays* L.), water-saturated and ground to a paste
Yellow commercial corn meal, cooked before sterilization
Rye (*Secale cereale* L.), whole, water-saturated
Rat ration pellets, wetted to point of crumbling.

Other investigators have reported the following media suitable for growing the fungus but the writer found them to be unsatisfactory in large scale production:

Potato (*Solanum tuberosum* L.), raw cubes and slices (Kalashnikov, 2)
Potato, boiled cubes and slices
Potato, cubes rinsed with 25 per cent molasses solution
Bran (one part by weight of bran, one part of water) (McCoy and Carver, 4).

Large amounts of bulk culture media were contaminated by certain fungi, mostly *Neurospora sitophila* Mont., *Aspergillus* spp., *Penicillium* spp. It was found that spraying a spore suspension on the media enabled very fast inoculation of the culture with minimum contamination resulting. The spore suspension was made by mixing two grams of Alconox with one gram of spores and adding 100 ml. of water.

Spore yield was lowered by high relative humidity which produced rapid germination and vegetative growth of the fungus but resulted in a low spore production rate. The ideal condition for spore production was found to be a very moist but not wet medium with a low humidity maintained after germination of the seeding spores. The effect on spore production of a continued high humidity is shown in Figure 1 D. The dish at the lower left had not been opened prior to being photographed. The dish at the lower right was permitted to dry out in air for a week prior to being photographed. The plate at the top of Figure 1 D was kept in a calcium chloride dessicating chamber for a week prior to being photographed. The media of the dishes were (top) rat ration pellets, wetted and crumbled; (lower left) corn kernels, water-saturated and ground; (lower right) Tendergreen bush beans, water-saturated and ground. Other tests have shown that the difference in media does not account for the variation in amount of spore production. Exposure of these well established cultures to air did not bring about contamination.

The fungus was cultured successfully in 9-cm. Petri dishes on bulk media. The prepared media were placed in the Petri dishes, autoclaved, and sprayed with a 1 per cent *B. bassiana* spore suspension. The fungus was cultured for about two weeks at 65° F. The hyphae completely bound the media and a small amount of sporulation resulted. These spores were collected with a camel's hair brush. The large fragments of media were broken up by hand into small lumps and the entire mass was replaced in the Petri dishes. The Petri dishes were placed in a high humidity for seven to ten days and then transferred to a low humidity for three to five days. Sporulation in large amounts took place. Collection of spores was made by sifting, accompanied with violent agitation. After spore collection the fragments were replaced in the Petri dishes, moistened, and cultured as before. Production by this method yielded 60 g. of *B. bassiana* spores per pound of dried beans. One pound of beans prepared enough media to fill 40 Petri dishes 9 cm. in diameter. The first sifting produced 1.0 g. of spores per dish, the second sifting 0.5 g.

Unsuccessful methods for bulk media culture included Erlenmeyer flask culture, cultivation on live vegetable matter, cultivation on whole seeds, and inoculation of large insects. The Erlenmeyer flask culture, although free of contamination, was unsuitable because the hyphae bound the media into a solid mass which was difficult to remove for the purpose of breaking into fragments for reculture. Cultivation on live vegetable matter did not produce large amounts of collectable spores. Cultivation on whole, water-saturated seeds resulted in a low yield per pound and a very slow rate of spore production. Inoculation of large insects was unsuccessful because of the failure of fungus sporulation in over 50 per cent of the infected insects.

LABORATORY AND FIELD EXPERIMENTS

Dusting and spraying were the methods of spore distribution used in the present experiments. Spraying was the most effective method but the amount of spores used was always much greater than employed in dusting. The effectiveness of sprays was greatly increased by the addition of the wetting agent, Alconox, which aided by maintaining the suspension of the spores in the water carrier. Alconox in solution has a pH of 9 to 10; this did not seem to be an inhibiting factor in germination of the spores.

Dusting was the method most used in these experiments. Dusts have the advantage of being able to be stored mixed, ready for use. The carrier ultimately used in all the dusts was wheat flour. In some tests, carrier dusts were made of finely ground, commercial anhydrous calcium chloride, which, it was thought, might hasten germination by attracting moisture to the area of the spore. On the contrary, it was found that germination was inhibited, due, possibly, to the alkalinity of the dust (pH 10) resulting from a calcium carbonate impurity.

Susceptibility to fungus infection was difficult to determine in the laboratory because of the presence of contaminating spores. At first, checks were run in the laboratory but sooner or later these became infected. To overcome this, counts were made of normal populations in the insectary or greenhouse.

EFFECTS ON INSECTS

In this study, many species of insects were exposed to the fungus in moist chamber or field tests to determine their susceptibility. Susceptible species are listed below. Species also reported by Charles (1) as susceptible to *B. bassiana* are marked by an asterisk.

Order and species or family:	Susceptible stage:
Orthoptera	
Periplaneta americana L.	Nymph
Dissosteira carolina L.	Adult
Locustidae	1st & 2nd instar nymph
Gryllidae	1st & 2nd instar nymph
Tettigoniidae	1st & 2nd instar nymph
Lepidoptera	
Prodenia eridania Cram.	Larva
Hyphantria cunea Drury	Larva
Protoparce quinquemaculata Haw.	Larva
Coleoptera	
Epilachna varivestis Muls.	Egg, larva, pupa, adult
Adalia bipunctata L.	Adult
Hippodamia 13-punctata L.	Adult
Coccinella trifasciata L.	Adult
Epicauta pennsylvanica DeGeer	Adult
Epicauta marginata Fabricius	Adult
Elateridae	Adult
Lema trilineata Oliv.	Adult
* *Leptinotarsa decemlineata* Say	Larva, pupa
* *Popillia japonica* Newm.	Adult
Tribolium confusum Duv.	Larva, adult
Sitophilus oryza L.	Adult
Acanthoscelides obtectus Say	Adult
Attagenus piceus Oliv.	Larva, adult
Rhynchites bicolor Fabr.	Adult
Homoptera	
Aphis rumicis L.	Nymph, adult
Macrosiphum rosae L.	Nymph, adult
Pseudococcus longispinus Targioni	Nymph, adult

Order and species or family:	Susceptible stage:
Hemiptera	
Anasa tristis DeG.	Nymph, adult
Thysanoptera	
Scirtothrips citri Moult.	Nymph, adult
Diptera	
Musca domestica L.	Egg, larva, adult
Arachnida	
Tetranychus bimaculatus Harvey	Nymph, adult

The representation on this list indicates that many insects are susceptible to *B. bassiana* though predatory and parasitic insects seem rarely liable to attack. The description of the infection of the European corn borer (*Pyrausta nubilalis* Hbn.) by *B. bassiana* as given by Lefebvre (3, p. 126) follows: " . . . the infected larvae turning pink. . . . The larvae soon become mummified, and after a few days a white mycelial outgrowth is evident, which turns to a creamy, powdery mass due to abundant spore formation."

In the experiments conducted by the author the common symptom was paralysis followed by death. Some insects were found to be completely paralyzed, in others all appendages except the mouthparts were paralyzed, or only one side of the body was paralyzed. Paralysis was followed by a subcuticular blackening. This stage represented death of the insect. Some of the forms liquefied at this point; others just dried up. It was the rare specimen in which luxuriant sporulation occurred. The photographs (Figs. 1, A and B, and 2) show some specimens on which a large amount of fungus spores has been produced. The amount of sporulation varied from none to the luxuriant growths shown in the photographs. These experiments showed that the greater portion of fungus-infected insects liquefied on death. This condition has been commonly associated with bacteria, but seldom with fungi. It is concluded that liquefaction is due to the histolysis of the insect tissues by the intestinal bacteria present after death induced by the fungus.

The following represent laboratory and field control tests on which data were recorded.

Rice grains were dusted with a small amount of pure spore material and 10 adult rice weevils (*Sitophilus oryza*) were introduced into the mixture. At temperatures of 65° to 85° F. and 35 to 85 per cent relative humidity the population was killed within 15 days. It required a concentration of 0.3 per cent by weight of spores to give a 100 per cent kill in 15 days. Lower concentrations tested were not effective.

Satisfactory control was obtained of the American roach, black bean aphid, rose aphid, and larvae of the Southern house mosquito in laboratory

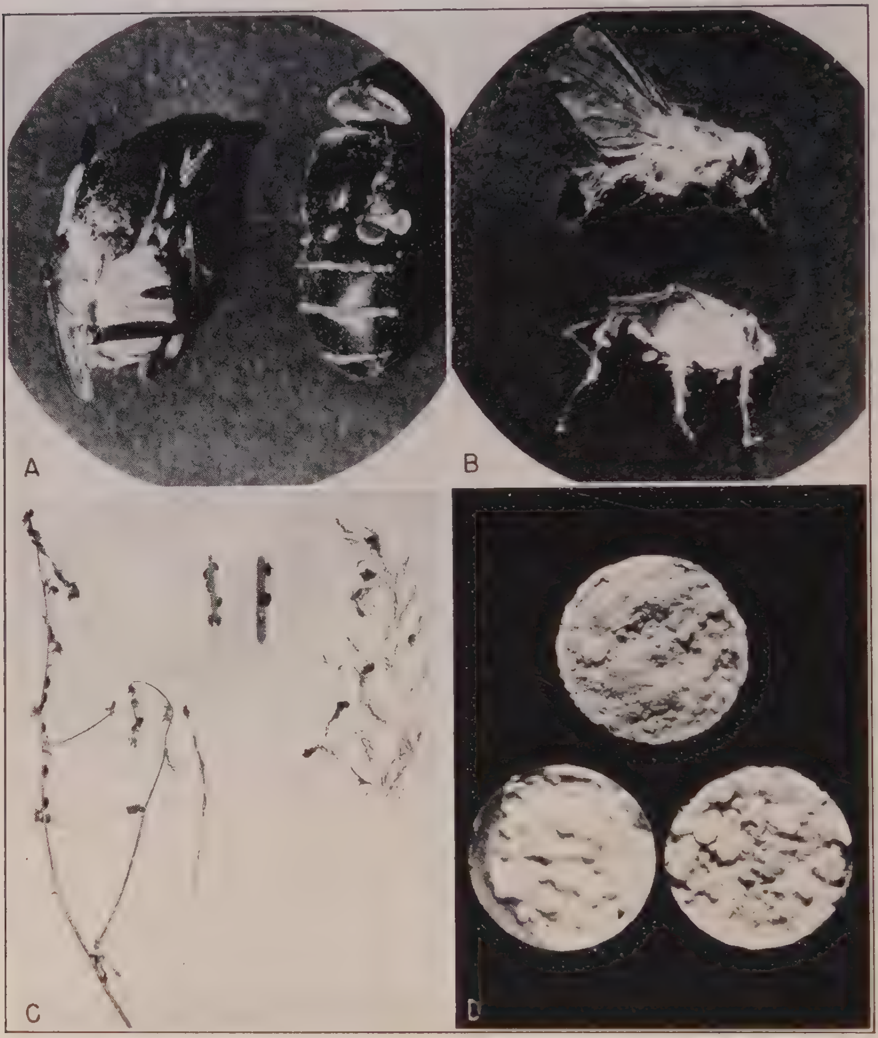

FIGURE 1. Effect of entomogenous fungi on flies; *Beauvaria bassiana* in Petri dish culture. A. Blowfly, *Lucilia unicolor*. Specimens from a large population which was wiped out by *Empusa americana* in the field. Note the tendency of the fungus to break out of the body cavity only along the conjunctivae. B. Houseflies attacked by *Beauvaria bassiana* in a moist chamber. C. Blowflies clinging to the stalks of chicory and grasses. D. *Beauvaria bassiana* in culture on media. (Top) Rat ration pellets, wetted and crumbled; (lower left) corn kernels, soaked and ground; (lower right) Tendergreen bush beans, soaked and ground. The intense differences in sporulation are due to humidity, not to difference in media.

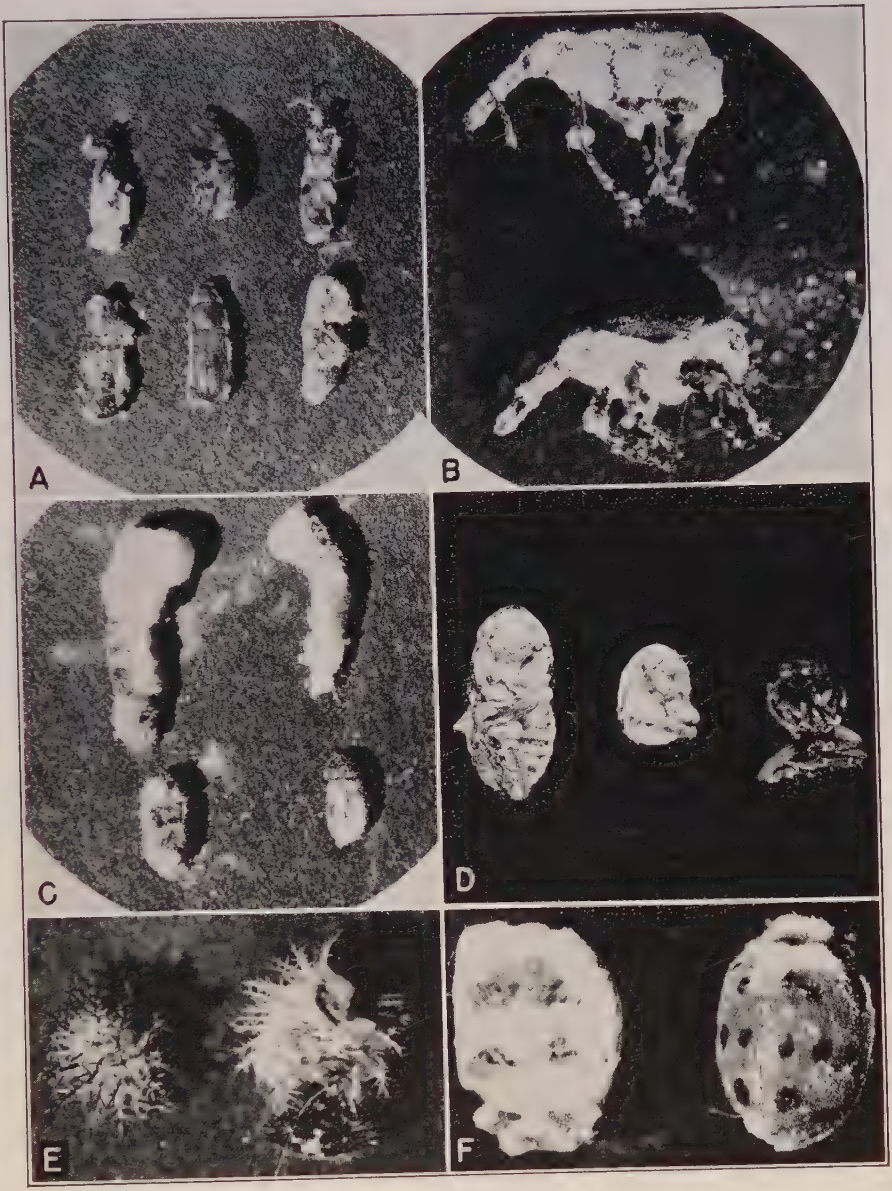

FIGURE 2. Insects subject to *Beauvaria bassiana* under moist chamber conditions. Note differences in amount of sporulation. A. (Top) Rice weevil; (bottom) confused flour beetle. B. Rose curculio, *Rhynchites bicolor*. C. Black carpet beetle, *Attagenus piceus*. (Top) Larvae; (bottom) adults. D. American roach nymphs. Note that the specimen at extreme right shows "liquefaction" rather than sporulation. E. Mexican bean beetle larvae. F. Mexican bean beetle adults. The fungus does not produce spores on the part of the body that has been resting on the ground.

TABLE I

EFFECT OF B. BASSIANA ON SURVIVAL OF FIVE SPECIES OF INSECTS IN LABORATORY AND FIELD TESTS

Common name	Scientific name	Stage of insect	Method of application of *B. bassiana* spores	Duration of test, days	Temp., ° F.	% Rel. humid.	No. of insects	% Dead in treated*
American roach	*Periplaneta americana*	Nymph (late instar)	Injected with 1% spore suspension	7	70–90	80– 95	10	100
			Fed rat pellets sprayed with 1% spore suspension	7	70–90	80– 95	20	100
			Dusted with spores	14	70–90	80– 95	10	100
Black bean aphid	*Aphis rumicis*	Agamic	Sprayed with 0.1% spore suspension	3	70–90	90–100	150+	100
Confused flour beetle	*Tribolium confusum*	Adults and larvae	Bran media estimated to contain 0.1% by wt. of spores	14	70–90	35– 95	18	46**
Rose aphid	*Macrosiphum rosae*	Agamic	Infested rose plants in field dusted with 0.5% spore dust	5	60–95	35– 95	100+	80†
Southern house mosquito	*Culex quinquefasciatus* Say	Larvae	Acetone extract of mycelium in bean substrate diluted in water to less than 200 p.p.m.	2/3	75–80	—	24	92

* Mortality in the check did not exceed 10%.

** Reduction in population as compared with check.

† All aphids had disappeared 5 days later.

and field tests. Control of the confused flour beetle was unsatisfactory (Table 1).

About 250 four-day-old houseflies (*Musca domestica*) were fed milk which had been dusted with 0.25 g. of 1 per cent *B. bassiana* spores. A second group of flies was not treated but was contaminated by air-borne fungus spores in the laboratory. At the end of 24 hours 37 per cent were dead in the fungus-dusted cage, 11 per cent in the fungus-contaminated cage, and less than 2 per cent in check cages in the insectary. Only the fungus-dusted milk dish contained no eggs. On the next feeding milk was added to the dishes and within 24 hours all the flies were dead in both the laboratory cages. A luxuriant fungus growth was evident on both the milk-soaked cotton pads, and the eggs in the contaminated dish were infected by the fungus. Less than 5 per cent of the insectary flies were dead.

Two cages of housefly pupae, about 250 each, were dusted with 1 per cent *B. bassiana* spores. There was no decrease in emergence as a consequence of this dusting. A battery jar containing bran-alfalfa mash with 3 cc. of housefly eggs was dusted with 3.0 g. of 0.5 per cent *B. bassiana* spores. There was no noticeable decrease in the numbers which completed their life cycle. The adults were kept in the rearing chamber but produced no eggs.

A moist chamber eight inches in diameter, with a piece of wet filter paper covering the bottom, humidity at condensation point, was dusted with 1.0 g. of 0.5 per cent *B. bassiana* spores. About 200 flies were liberated in the chamber and after a half hour the flies were dropping with either partial or complete paralysis. Within three hours there was 100 per cent knockdown with no subsequent recovery. These results were confirmed in two repetitions of the test. This same experiment repeated at 70 to 80 per cent relative humidity gave neither knockdown nor decrease in egg production. From these observations it has been concluded that the housefly is susceptible to paralysis caused by the fungus when it is in a liquid medium.

Field tests with the two-spotted spider mite (*Tetranychus bimaculatus*) on beans showed a large number killed due to dusting with 0.5 per cent *B. bassiana* spores. During the period of the test, July 16 to July 21, the temperature and relative humidity were well within the optimum range for the fungus. The temperature averaged 74° F. and the relative humidity at 7:30 A.M. averaged 91.3 per cent. Some rain fell during three of the five days of the test. The normal insectary mortality of mites was 5 to 10 per cent. Some of the dead checks showed symptoms of fungus infection. The checks were located ten yards downwind from the test plants which may have accounted for the fungus contamination. Many of the dead showed the usual symptoms, liquefaction or mycelial growth. It is thought the kill was higher than actually represented by the data because some of the leaves with low population counts exhibited injury that could have been made only by large numbers of mites. The dead were easily shaken off the leaves (Table II).

TABLE II

EFFECT OF FIVE DAYS' EXPOSURE TO 0.5 PER CENT B. BASSIANA SPORE DUST ON SURVIVAL OF TWO-SPOTTED SPIDER MITE ON BUSH BEAN PLANTS IN THE FIELD

Dusted		Control	
Number of mites on leaf	Per cent dead	Number of mites on leaf	Per cent dead
48	42	4	75
15	73	10	70
14	71	29	34
13	100	67	6
8	100	1	0
12	75	4	50
34	40	30	20
14	79	1	0
13	76	1	0
18	72	49	4
6	50	14	22
11	81		
5	100		
7	43		
7	86		
14	50		
Total 239	Av. 71.1	Total 210	Av. 25.5

Both laboratory and field tests were made with the Mexican bean beetle (*Epilachna varivestis*). It was noted that all stages were susceptible to *B. bassiana*. In a field test, eggs laid on the plants by the adults before the plants were dusted were killed by the fungus before hatching (Table III). Cultures of egg clusters in Petri dishes showed an even greater susceptibility to the effects of the fungus. Dusting with 0.5 per cent *B. bassiana* spores

TABLE III

EFFECT OF 0.5 PER CENT B. BASSIANA SPORE DUST ON SURVIVAL OF MEXICAN BEAN BEETLE POPULATIONS ON BUSH BEANS IN THE FIELD EXPRESSED AS THE NUMBER SURVIVING

Stage of development	Number surviving after various intervals				
	First planting			Second planting	
	At start	5 days	10 days	At start	5 days
Adults	20	3	2	10	3
Larvae	10	2*	0**	20	1
Egg clusters	1	0	0	—	—

* Also 1 pupa alive.
** No pupa alive.

resulted in some liquefaction of the egg clusters within 18 hours and 100 per cent liquefaction within 72 hours after dusting. Bean beetle eggs require a high humidity for hatching; this same high humidity is most favorable for the germination and development of the fungus. Early in August it was noted that the greenhouse culture was lightly infected with fungus, probably *B. bassiana.* This resulted in about 50 per cent mortality of egg clusters and 5 per cent mortality of adults. No dead larvae were noted. A few pupae were also infected.

In a laboratory test of 17 late instar larvae dusted with *B. bassiana* spores (100 per cent) in Petri dishes, nine died as larvae, and eight transformed to the pupal stage before dying. Of the control all but one transformed to adults. A field test on 12 pots of beans artificially infested with 38 bean beetle adults showed that 100 per cent of the adults present were attacked by the fungus when dusted with 0.5 per cent of *B. bassiana* spores. The fact that a large number of adults in the check were killed by the fungus is an indication of both the spread of the fungus by drift, and its potency even in much lower concentrations. The checks were located ten yards downwind of the test plants. At the time of this test there were no insects killed by the fungus in the insectary population. A field test was made using a flat of 12 bean plants with artificially established adult and larval populations. This was an attempt to determine whether the losses in unrecovered specimens were due to the adults being repelled by the dust and flying away. The larvae used included all stages from newly hatched to the late instars. The larvae as well as the adults died or disappeared (Table III). The insectary culture at this time showed no loss of either larvae or adults.

The Mexican bean beetle control effected by dusting plots with 1 per cent of *B. bassiana* spores at a rate of 20 lbs. per acre was consistent with the preliminary results obtained in the small scale indoor and outdoor tests. The spore dust was applied in two applications, each at the rate of 10 lbs. per acre. The rotenone was applied in one 20-lb. per acre application on August 25. The spore applications were made on August 24 and 26, the last dusting being completed about one hour before the start of a 3.69-inch rainfall. The count was made 14 days after dusting was completed (Table IV). The dispersal characteristic of the fungus made it imperative that the various plots be widely separated. The fungus-dusted plot was between the other two plots; the untreated area was about one mile away; the rotenone-dusted area was about one-quarter mile from the spore-treated area. All these areas had been planted to beans for almost the entire summer. The old neglected plants had been partly devoured by the large beetle populations. Very few fungus-killed specimens were found in the treated areas. The excessive rains that were common during the period of testing, August 26 to September 11, could account for washing away many of the dead forms. Predatory or scavenger insects may also have been aided in their search for food by the effect of the fungus in paralyzing the host beetles. It was noted that *Podisus maculiventris* Say, adults and nymphs, were feeding on the larvae in both fungus-dusted and check plots. None was found in the rotenone-dusted areas. The poor showing of the rotenone-treated plots can be accounted for by the almost daily rains. It was these same rains which increased the effectiveness of the artificially spread *B. bassiana* spores.

TABLE IV

EFFECT OF 14 DAYS' EXPOSURE TO 20 LBS. PER ACRE OF 1 PER CENT B. BASSIANA SPORE DUST AND 0.75 PER CENT ROTENONE DUST ON MEXICAN BEAN BEETLE FIELD POPULATIONS

Treatment and plot description	Average number of insects per 10 ft. row				Estimated injury, per cent
	Egg clusters	Feeding larvae	Pupae and post-feeding larvae	Adults	
B. bassiana on 9-week-old bush beans. 100 ft. row	0	0.6	1.4	1.2	—
B. bassiana on 9-week-old lima beans. 120 ft. row	0	1.1	0.9	0.5	—
B. bassiana on 6-week-old bush beans. 120 ft. row	0.6	8.3	1.8	1.2	0–10
Rotenone on 6-week-old bush beans. 30 ft. row	1.7	28.3	15.7	4.0	25
Control 6-week-old bush beans. 10 ft. row	10.0	87.0	168.0	76.0	90

EFFECTS ON ANIMALS OTHER THAN INSECTS

In handling the sporulated material, especially when separating the spores, small clouds of dust (spores) were often inhaled. This did not have any harmful effect on the operator.

A suspension of 0.5 cc. of 1.0 per cent *B. bassiana* spores was injected into the abdominal cavity of a rat (*Rattus norvegicus* L.), with no adverse after-effects. A suspension of 0.25 cc. of 1.0 per cent of *B. bassiana* spores was injected into the muscle (right shoulder) of a rat. No pathological symptoms developed. Two rats were fed 0.5 g. of *B. bassiana* spores added to bran and molasses. This diet produced no adverse effect.

Slugs (*Limax* sp.) dusted in a moist chamber were killed by *B. bassiana*. The symptoms of the disease were partial liquefaction followed by sporulation of the fungus. The action of the fungus was extremely slow requiring five days to two weeks to take effect.

EFFECTS ON PLANTS AND FRUITS

When *B. bassiana* spores were sprayed or dusted onto normal plant material, there were no serious effects. When placed on bean plants, it was noted that some of the spores germinated and sporulated on the hairs of the underside of the leaf. This was not as extensive nor as harmful to the leaf as bean mildew fungus. When spores were sprayed or dusted onto leaves removed from the stem and kept in a moist chamber, the fungus germinated, grew, and sporulated. A liquefaction of the plant tissue in the area of growth was noted. *B. bassiana* spores, 0.5 per cent, dusted on an orange fruit (*Citrus sinensis* Osbeck) to control thrips showed little effect after three days; after six days the entire orange was covered with a heavy growth of *B. bassiana* very similar to that exhibited by *Penicillium* spp. The thrips were also covered with fungus. Grasses and weeds on which insects had died were not affected by the fungus although the humidity was optimum for spore germination.

NATURAL OUTBREAKS OF FUNGUS DISEASES OF INSECTS

Most recorded spontaneous outbreaks that have reduced or eliminated insect populations have occurred in the spring or autumn. Entomogenous fungi require two basic conditions for mass attack: a high relative humidity and a heavy insect population. The highest percentage germination of the spores occurs at the dew point. This is a confirmation of Pascalet's (6) report which stated that the most effective form of high humidity is a light rain or fog. Under conditions of over 90 per cent relative humidity spore germination took place very quickly resulting in the paralysis of houseflies after one-half hour exposure to the fungus spores. With optimum conditions for fungus growth large groups of insects can be controlled in very short periods. Rains of ten minutes to one-half hour satisfy this requirement. In the New York area during the months of June, July and August 1947 there were 8 to 12 days each month during which rain fell according to the weather records of U. S. Dept. of Commerce Weather Bureau in New York City (8). These showers coupled with the long periods of 90 per cent relative humidity in the dawn hours insured the effectiveness of applied fungus control in this area.

Two natural outbreaks of fungus affecting blowflies were observed, and were correlated with periods of high humidity accompanied by frequent showers. In only one instance was a large population of flies present. The first outbreak of fungus disease was found on a mink ranch near Ithaca, N. Y. during July. The fungus was identified as *Empusa americana* Thax. by Dr. J. A. Stevenson, Bureau of Plant Industry, Beltsville, Md. The blowfly was identified as *Lucilia unicolor* Towns. by Dr. Roy Melvin, Westvaco Biological Laboratory, Boyce Thompson Institute. The flies were breeding in food droppings from the mink cages. The food was composed of about 75 per cent meat and 25 per cent of a vegetable mixture. Previous to the outbreak it was estimated that there were 200 to 300 flies constantly buzzing about each of 900 cages annoying both the animals and the operators.

On July 1, dead flies were first noted clinging to the stalks of grasses, legumes, and weeds (Fig. 1 C). During the first days about 30 per cent of the flies died; at the end of two weeks about 60 per cent had been killed by the fungus; by August 1 almost 100 per cent of the flies had disappeared. From August 1 until September 20 practically no flies were seen. During an eight-hour period only 10 flies were encountered in the mink yard. No other large flies, such as house or stable flies, were noted at this time. Examination of the droppings under the cages showed maggots developing in only two; both of these were under new cages. This natural infestation occurred after one month of frequent rains, high relative humidity, and optimum temperatures for fungus development. The one-month period, July 1 to August 1, from the first notice of the fungus-killed flies in the field until almost 100 per cent control had been accomplished, included 17 days of some rain. The Ithaca area weather data are taken from the monthly meteorological summary, Agronomy Dept., Cornell University (5).

The absence of all other large species of saprophytic and coprophytic Diptera indicates that these other species may also be controlled by this fungus. The presence of small numbers of flies, possibly migrants, and the virtual absence of maggot cultures indicate that either the fungus produces sterility or the fungus growing on the droppings destroys immature stages preventing fly development. The possibility of this fungus, *Empusa americana*, being in saprophytic culture in the field is borne out by the fact that it was cultured on media in the laboratory. An entomogenous fungus, capable of killing houseflies, was isolated from specimens of the flies collected. The fungus was cultured on potato-dextrose agar and molasses agar. It was noted that spore production of *Empusa americana* was about as good as *B. bassiana*. Tests with houseflies under optimum conditions showed that *E. americana* was much less effective than *B. bassiana*. *B. bassiana* controlled the flies in three hours under moist chamber conditions; with 80 to 90 per cent relative humidity, 100 per cent kill was obtained in 24 hours. Under moist chamber conditions *E. americana* produced only 50 per cent kill in 24 hours with the remainder of the flies living 72 hours. For this reason further tests with *E. americana* were omitted.

The second outbreak of fungus affecting blowflies was noted in the vicinity of Kingston, R. I. The area was a small chicken yard. The population of flies was very sparse with less than ten live specimens observed. In all, ten infected specimens were found. The flies and fungus in this outbreak seemed to be identical with those collected in the Ithaca outbreak. Again, the high humidity and frequent rains in this area previous to the outbreak were optimum for fungus spore germination and development. It is of interest to note that with this infestation no large population of flies was present that summer.

The conclusion drawn from these observations is that a natural outbreak requires a long period of optimum conditions for incubation of sufficient numbers of spores to infest the entire population. Complete control by natural outbreaks requires optimum conditions of exceptional duration. The long incubation period that prevailed during the fungus outbreak at Ithaca indicates that by natural means it took many weeks for the infecting fungus to reach epidemic proportions. Artificial spread of the fungus spore during the period before July 1 would have been advantageous in speeding up the control of the blowfly infestation.

EFFECT OF BEAUVARIA BASSIANA ON BIOLOGICAL CONTROL AGENTS

One of the disadvantages of the use of insecticides is the indiscriminate killing of both pest insects and biological control agents. For this reason special attention was directed to the effects that the fungus had on insect parasites and predators.

Four maggots of *Muscina stabulans* Fall.[3] were found feeding within and on the body of a late instar American roach infected with *B. bassiana*. The roach had been segregated since June 10 from the open container in which it had been raised. On June 17 the roach was dead, presumably due to the effect of the established fungus though possibly due to the effect of the maggots. Other roaches not parasitized by maggots were killed by the fungus. A fresh, decapitated roach was substituted for the fungus-killed host. At this time the maggots were less than one-eighth inch long. These maggots pupated on June 30; the flies emerged on July 4 and died within six days, perhaps because they were kept in a fungus-contaminated flask.

Two pupae of *Compsilura concinnata* Bousche[3] were recovered from the body of a tomato hornworm which had been killed by fungus dusting. The adult flies emerged five days after pupating. One pupa was recovered from a mass of Japanese beetle adults which had been treated with and died from *B. bassiana*. The adult emerged about five days later.

Field tests of the effect of *B. bassiana* dust, 0.5 per cent, on *Macrosiphum rosae* showed considerable competition for the control of aphids by Syrphidae and Chrysopidae larvae. Many eggs, young larvae, and adults were observed; none of these forms seemed to be in the least affected by the fungus dust. Most of the control can be attributed to the predators rather than the fungus. The fungus was present in large amount and under humidity conditions which caused some infection of the aphids.

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[3] Identified by Dr. C. H. Curran, American Museum of Natural History, New York, N. Y.

Externally pupating parasites (*Apanteles congregatus* Say) of the tomato hornworm treated with less than 5 per cent *B. bassiana* dust were not killed. These parasites which were larvae or pupae at the time of dusting completed development and emerged. The host tomato hornworms were killed by the fungus.

Field tests showed that bean beetle populations were attacked, but not controlled, by the predator, *Podisus maculiventris* Say.[4] The population of bean beetles was definitely controlled by the fungus, some infected beetles were found, yet the predator was still there feeding. In regard to these predators the only claim that can be made against the fungus is the competitive effect between the two. These tests were all made during a period of optimum humidity for fungus spore germination.

Laboratory tests showed that ladybird beetle adults (Coccinellidae) were susceptible to *B. bassiana*. The effect of this fungus on Mexican bean beetles is probably an indication of the effect on these predatory beetles as they belong to the same family.

SUMMARY AND CONCLUSIONS

A study was made of the relationship of entomogenous fungi to insect control. The species of fungi tested were: *Metarrhizum glutinosum* Pope, *Empusa americana* Thax., and *Beauvaria bassiana* (Bals.) Vuill. Preliminary tests showed that *B. bassiana* was the most spore-productive in artificial culture and the most effective for insect control of the three species under consideration. Because of these advantages, subsequent tests were made only with *B. bassiana*. Tests with *B. bassiana* included data relating to the range of susceptible host species, moisture requirements, artificial culture methods, laboratory and field applications of the spores, and the effect on biological control agents.

Large quantities of *B. bassiana* spores were produced in artificial culture on a medium of bush beans. Production of the spores was at the rate of 60 grams per pound of beans or enough for dusting about three acres. The method of culture is practical for large scale production.

When *B. bassiana* attacked an insect, the primary symptoms were paralysis and death accompanied by changes in the gross appearance of the infected insect. The changes were emergence of the mycelium from the body with its subsequent spore production or liquefaction of the insect body without any spore production. The effect of *B. bassiana* was not entirely due to parasitism but was in part due to a constituent of the mycelium. This constituent is capable of causing death to mosquito larvae at less than 200 p.p.m.

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[4] Identified by Dr. Charles Reichart, Ohio State University, Columbus, Ohio.

Laboratory tests indicated that most insects were susceptible to attack by *B. bassiana* and that heavy spore concentrations of *B. bassiana* under high humidity conditions at normal temperature will control insects found in stored products. Field tests of *B. bassiana* compared with the use of rotenone suggested that the fungus was better at controlling the Mexican bean beetle under the frequent heavy rainfall conditions common in New York during the test period.

A natural outbreak of *Empusa americana* Thax. which controlled the infestation of the blowfly, *Lucilia unicolor* Towns., was observed in Ithaca, N. Y. It is suggested that the long preceding period of rainfall was necessary for the disease to build up to epidemic proportions.

The main limiting ecological factor in the use of artificially spread fungus spores was atmospheric humidity. The best form of moisture was rainfall, an optimum period of about one-half hour being sufficient to cause infection of the insects. This optimum humidity was present at the time of the observations reported here and is common each summer in the lower New York area. However, humidity alone is not sufficient to insure natural control of insects. It is believed that entomogenous fungi can be depended upon to control insect pest infestations in the frequent rainfall and high humidity areas of the Atlantic Coast of the United States. Areas outside of this weather pattern should not depend on entomogenous fungi for the control of pest populations. If the exacting requirements for entomogenous fungi are met, an inexpensive, long-lasting control is likely to result.

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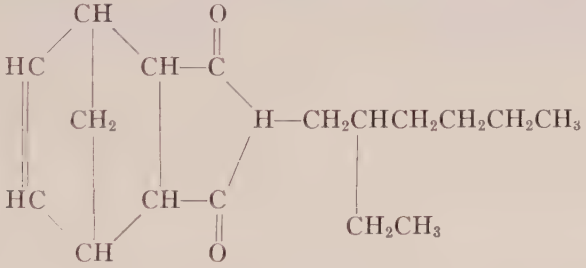
EFFECTIVENESS OF *N*-(2-ETHYLHEXYL)BICYCLO[2.2.1]-5-HEPTENE-2,3-DICARBOXIMIDE ON HOUSEFLIES

ALBERT HARTZELL

N-(2-ethylhexyl)bicyclo[2.2.1]-5-heptene-2,3-dicarboximide is now commercially available as an insecticide.[1] The performance of aerosol bombs containing this compound plus other toxicants has been reported by Nelson *et al.* (4). Other *N*-alkyl imides derived from the same parent bicyclo[2.2.1]-5-heptene-2,3-dicarboxylic acid have been reported in the literature. The *n*-butyl imide has been patented by McCrone (3), and the amyl imide by Bousquet (1, 2). The insecticidal, miticidal, and repellent properties of other *N*-alkyl imides have been reported by other workers at the Orlando Laboratory of the United States Department of Agriculture (6, item Nos. 647, 1374, 2288, 3747, 4641, 4794, 5123, and 6036).

CHEMICAL AND PHYSICAL NATURE OF INSECTICIDAL COMPOUND

Preparation. The following data were supplied by Dr. A. A. Schreiber of Van Dyk & Company, Inc., Belleville, New Jersey. The chemical name of the insecticidal compound is *N*-(2-ethylhexyl)bicyclo[2.2.1]-5-heptene-2,3-dicarboximide and is represented by the formula:



It is easily prepared with a high yield by first condensing cyclopentadine and maleic anhydride in the usual manner of the Diels-Alder synthesis and heating the resulting anhydride with about an equimolar quantity of 2-ethylhexylamine in the presence of a solvent of the benzene series until the stoichiometric amount of water has been removed. The commercial product has a purity of about 99 per cent. The chief impurity is 2-ethylhexanol.

Physical properties.

Boiling point, 158 ± 2° C., at 2 mm., uncor.; decomposes on distillation at room temperature.

Specific gravity at 18° C., 1.05.

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[1] Sold under the trade name "Van Dyk 264," formerly designated as "Octacide 264."

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Refraction index, η_D^{20}, 1.505 ± 0.005.

Flash point, above 350° F.

Completely miscible with petroleum fractions, halogenated hydrocarbons, methylene chloride, the Freons, and Genetrons. Insecticidal compounds such as DDT, benzene hexachloride, etc., are very soluble in this compound.

Anal. Calcd. for $C_{17}H_{25}O_2N$: N, 5.08 per cent. Found: N, 5.00 per cent.

METHODS OF TESTING

The compound was tested against houseflies (*Musca domestica* L.) by the large group Peet-Grady method as recommended by the National Association of Insecticide and Disinfectant Manufacturers (5). The regular Official Test Insecticide (O.T.I.) supplied by the above Association and which contains approximately 0.1 g. pyrethrins per 100 ml. was used in control tests against each culture of flies used. Test solutions were prepared by dissolving the compound in "Deo-base" (a purified petroleum distillate). In some cases pyrethrins extract equivalent to 0.025 g. pyrethrins per 100 ml. was included in the test solutions to provide adequate knockdown.

RESULTS

The results of tests with this compound alone and in combination with pyrethrins, DDT, and Chlordan appear in Table I. It will be noted that

TABLE I

RESULTS OF PEET-GRADY TESTS WITH *N*-(2-ETHYLHEXYL)BICYCLO[2.2.1]-5-HEPTENE-2,3-DICARBOXIMIDE AND WITH ADDED PYRETHRINS AND OTHER INSECTICIDES

Compound, %	Pyrethrins, %	Other insecticides	10 Min. knockdown, %	24 Hr. kill, %	O.T.I.	
					10 Min. knockdown, %	24 Hr. kill, %
0.8	0.02	0.2% DDT	89	85	88, 91	30, 39
1.0	0.02	0.25% DDT	94	82		
0.8	0.025	0.2% DDT	96	93	97, 99	43.5, 37.7
0.8	0.02	0.2% Chlordan	99	98		
1.0	0.02	0.2% DDT	94	93	91, 99	40.8, 51.1
0.8	0.02	0.2% DDT	92	92	94, 94	39.9, 44.2
0.8	0.025	0.2% DDT	92	92		
1.0	0.025	0.2% DDT	92	93		
0.8	0.02	0.2% DDT	93	91	98, 97	40.9, 42.2
2.0	—		77.8	67.3	85, 87	47, 54
2.0	0.025		94.4	67.6		
4.0	—		80.7	80.3		
4.0	0.025		93.7	91.4		
2.0	—		85.6	56.3	97, 95	44.7, 52.1
2.0	0.025		97.2	64.9		
4.0	—		93.8	81.5		
4.0	0.025		97.7	85		
—	0.025		84.0	21	95	50

it requires a concentration of 2 per cent of the compound plus 0.025 per cent pyrethrins to give satisfactory kill and knockdown of houseflies. When 0.2 per cent of DDT or Chlordan was added, the concentration of the compound could be reduced to 1 per cent and a satisfactory kill and knockdown obtained.

Low pressure aerosols in commercial cans were tested with formulations corresponding to those used by Nelson *et al.* (4) but at the rate of from 3 to 5 g. per 1000 cu. ft. A 10-minute knockdown and 24-hour kill of from 91 to 97 per cent have been obtained.

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SEED PACKETS AND ONION SEED VIABILITY[1]

Lela V. Barton

It has been well established that moisture content and temperature determine, to a large extent, the keeping quality of seeds in storage. Neither moisture nor temperature can be considered alone, since the effect of one depends upon the other. These factors not only affect seeds while they are in controlled storage rooms maintained by the seedsmen, but continue to exert a vital influence after they are removed and packeted for the trade. Two previous reports from this laboratory have demonstrated the rapid deterioration at unfavorable temperatures and humidities of onion seed upon removal from storage (1, 2). Furthermore, it is essential that the seeds maintain full viability under the original storage condition, in order to withstand further storage under unfavorable conditions. The better the storage condition for keeping the seeds viable, the better the chance of survival when they are removed to an unfavorable environment. Other conditions being equal, low temperature has been found to be superior to higher temperatures for the maintenance of germination capacity. However, seeds dried and stored sealed at ordinary room temperature may keep better than those stored open in a room at 5° C. where high humidity prevails, as has been demonstrated repeatedly in this laboratory. In these cases, then, resistance to further storage would be in favor of the original room temperature storage.

The keeping quality of any particular lot of seeds does not depend upon its initial high quality, but upon the storage conditions. Seeds of low germination capacity may be stored successfully for fairly long periods if storage is favorable. On the other hand, seeds in which deterioration has been initiated, even if the germination capacity is still high, are incapable of remaining viable for long periods under adverse storage conditions.

All of these facts point to the necessity of maintaining rooms in which temperature and humidity can be controlled if commercially-important seeds are to be held over for sale in subsequent years. Responsible seedsmen have come to realize this and have provided the proper facilities. However, another problem, that of deterioration upon removal from storage, is being recognized as of the utmost significance. Seedsmen must packet their seeds for the trade well in advance of the time the consumer buys and plants them. Kraft paper envelopes are commonly used, and are sent to wholesale and retail dealers all over the country. Some of these packeted

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[1] Most of this research was sponsored by the W. Atlee Burpee Co., Seed Growers, Philadelphia 32, Pa., and was conducted at Boyce Thompson Institute for Plant Research, Inc., Yonkers 3, New York.

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seeds are stored in warehouses or retail stores under conditions of high temperature or humidity or both where germination capacity decreases at such a rapid rate that the seeds may be worthless when the customer finally plants them.

Since moisture content of the seeds is such an important factor in their deterioration and since the paper packets commonly used in the trade are not in any sense waterproof, it became of interest to determine the difference in viability of seeds in these packets as compared with waterproof packets. Results to be presented below have demonstrated the superiority of tin cans with tight-fitting lids, or of heat-sealed aluminum foil envelopes over the ordinary kraft paper seed envelope for the successful packeting of onion seeds.

MATERIAL AND METHODS

Seeds of onion (*Allium cepa* L. variety Ebenezer) from Caldwell, Idaho, were received in this laboratory October 15, 1945. Germination tests and moisture determinations were made immediately. The moisture content of two portions of the seeds was adjusted to 10.6 and 3.5 per cent of the dry weight of the seeds by placing the seeds in desiccators over water or calcium oxide until the desired weight was obtained. The seeds were then stored on November 5, 1945. Storage was made at 5° and 25° C. in open containers and in sealed containers.

Viability tests were made after 3, 6, 12, and 24 months of storage. At each of these periods seeds from each storage condition were packeted in paper seed envelopes of the type usually used in the trade, and in tin cans with tight-fitting lids sealed with sealing wax.

These packets and tin cans were placed for further storage in a room at 30° C. The paper packets were stored in a desiccator over a saturated solution of sodium chloride giving approximately 76 per cent relative humidity. Five packets and five tin cans of seeds from each storage condition were prepared at the end of each of the four storage periods to allow for testing after two weeks to six months at 30° C. In a few cases, seeds from packets and tin cans were also tested after 9 and 12 months.

At each testing period duplicates of 100 seeds each were germinated on moist filter paper in Petri dishes at 20° C. and the same number of seeds in the greenhouse in a mixture of sand, sod soil, and granulated peat moss in equal parts.

RESULTS

KRAFT PAPER PACKETS VS. TIN CANS

In Table I are shown the germination percentages obtained, both at 20° C. and in the greenhouse, from seeds packeted after original storage periods of 3, 6, 12, and 24 months at 5° and 25° C. and then held for

TABLE I

GERMINATION OF STORED ONION SEEDS AFTER PACKETING AND FURTHER STORAGE

Germination condition	Original storage condition				% Germination*	% Germination after further storage in packets at 30° C., 76% relative humidity									
	Time, mos.	Temp., ° C.	Open or sealed	% Moisture		Paper packets (months)					Tin cans (months)				
						0.5	1	2	3	6	0.5	1	2	3	6
Moist filter paper at 20° C.	3	25	O S S	10.6 3.5	97 96 98	— — —	— — —	62 41 63	36 14 36	1 0 1	— — —	— — —	95 94 96	91 91 95	97 97 97
		5	O S S	10.6 3.5	97 97 98	— — —	— — —	64 67 62	29 38 44	0 2 1	— — —	— — —	96 92 94	95 95 95	97 91 97
	6	25	O S S	10.6 3.5	97 91 97	94 92 90	93 77 89	— — —	33 3 45	0 0 1	96 93 95	98 94 94	— — —	95 93 96	97 95 97
		5	O S S	10.6 3.5	94 93 91	93 96 95	92 94 95	— — —	40 38 45	1 1 1	99 91 97	95 97 93	— — —	97 94 98	93 96 96
	12	25	O S S	10.6 3.5	98 93 99	94 86 92	93 79 94	72 6 82	53 2 63	8 1 24	94 90 96	96 94 96	93 75 92	92 77 94	96 83 93
		5	O S S	10.6 3.5	95 96 98	98 94 92	93 96 95	78 86 77	57 55 56	16 25 26	97 97 97	98 96 99	96 94 98	94 93 94	92 95 91
	24	25	O S S	10.6 3.5	92 76 91	87 24 84	88 2 82	14 0 32	0 0 2	0 0 0	89 62 90	93 71 90	90 61 89	84 53 84	90 59 94
		5	O S S	10.6 3.5	96 95 97	88 95 94	93 95 90	17 26 29	0 1 6	0 0 0	92 92 95	97 94 96	96 97 95	93 94 92	92 96 93
Soil in greenhouse	3	25	O S S	10.6 3.5	88 45 56	— — —	— — —	33 20 38	7 4 10	0 0 0	— — —	— — —	83 79 84	89 76 84	78 45 60
		5	O S S	10.6 3.5	84 71 68	— — —	— — —	29 31 28	12 8 17	0 0 0	— — —	— — —	78 84 83	86 85 85	71 73 67
	6	25	O S S	10.6 3.5	91 87 85	85 81 77	79 64 81	— — —	2 0 5	0 0 0	93 81 84	83 85 84	— — —	77 81 77	80 68 74
		5	O S S	10.6 3.5	83 80 84	87 84 87	83 71 84	— — —	2 7 5	0 0 0	89 93 72	88 81 85	— — —	87 77 80	80 75 83
	12	25	O S S	10.6 3.5	85 75 83	93 57 87	70 36 85	61 2 72	25 0 28	1 0 3	88 61 90	84 62 90	84 59 85	78 52 85	81 52 72
		5	O S S	10.6 3.5	77 92 77	82 85 86	78 85 80	64 62 76	25 33 26	3 5 9	87 84 90	90 88 91	85 90 88	85 76 78	79 76 82
	24	25	O S S	10.6 3.5	86 44 81	81 7 81	68 0 63	12 0 9	1 0 1	0 0 0	86 40 83	81 47 86	86 37 86	81 28 87	77 17 89
		5	O S S	10.6 3.5	89 85 94	79 69 86	64 73 80	7 17 32	0 0 0	0 0 0	89 69 89	83 79 85	91 87 89	81 91 86	77 77 84

* Per cent germination upon removal from original storage condition for packeting.

additional periods of 2 weeks to 6 months at 30° C. and 76 per cent relative humidity.

It will be seen that complete capacity to germinate at 20° C. was maintained under all the conditions of the original storage up to and including 12 months. This means that for storage periods of two years or less, temperatures of 5° and 25° C., and open and sealed containers were equally effective in maintaining a high quality in the seeds as measured by their ability to germinate immediately upon removal from storage. Furthermore, there appeared to be no advantage in the initial drying of the seeds from 10.6 to 3.5 per cent moisture.

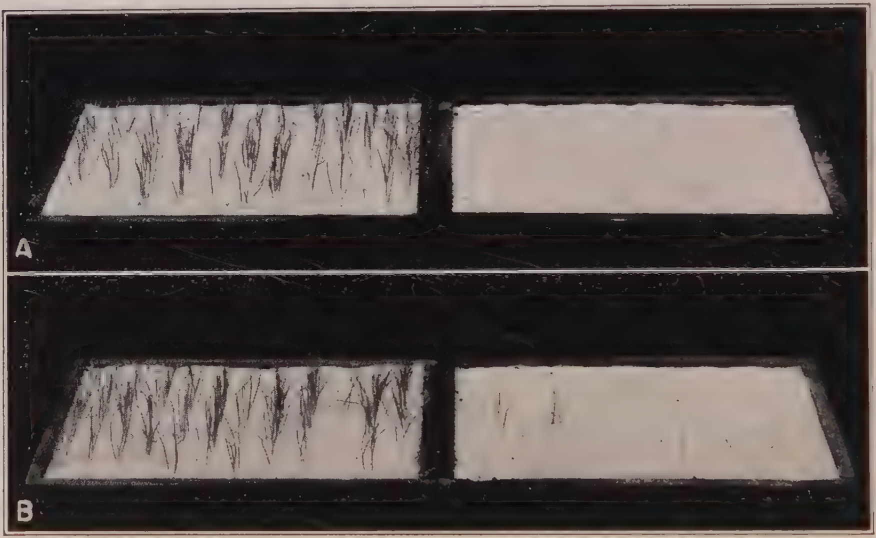

FIGURE 1. Seedling production from onion seeds held six months at 25° C. (A) or 5° C. (B), and then stored for an additional period of three months at 30° C. and 76 per cent relative humidity in tin cans (flats on left) or paper seed packets (flats on right).

An extension of the storage period to 24 months, however, proved deleterious to the onion seeds containing 10.6 per cent moisture when the storage temperature was 25° C. This will be seen in the reduction of the germination at 20° C. to 76 per cent. With this one exception, the germination capacity of all of the seed lots immediately upon removal from the original storage conditions was high, ranging from 91 to 99 per cent. That there were differences in these seeds as regards their ability to withstand further unfavorable storage will be shown below.

The advantage of open storage at 25° C. over sealed storage of seeds containing 10.6 per cent moisture is evident. The atmosphere in the 25° C. storage chamber was dry enough to permit a moisture content lower than

10.6 per cent for the seeds in open containers. It is always worse to seal seeds with excess moisture than to leave them in open containers in atmospheres of low humidity. In high-humidity atmospheres, however, the advantage of sealing even with 10.6 per cent moisture is apparent from the results of further storage at 76 per cent relative humidity.

Reference to the data secured by testing these same seeds after further storage at 30° C. and 76 per cent relative humidity reveals the striking superiority of sealed tin cans over the regulation seed packets (Table I). Six months after packeting in tin cans, full capacity to germinate on moist filter paper at 20° C. was retained by all lots regardless of the original storage conditions or time except the one with reduced germination at the time of packeting and even that lot still gave 59 per cent germination. An extension of the time in tin cans at 30° C. to twelve months failed to lower appreciably the germination capacity of onion seeds which had received a previous storage of six months at 5° or 25° C. This is in striking contrast to the fate of the seeds in paper packets, all of which were entirely worthless in six months and were so reduced in germination vigor after three months in the packets that good seedling stands could not be obtained in soil (Fig. 1).

Most rapid degeneration in paper seed packets is seen for the seeds containing an original 10.6 per cent moisture and from an original storage temperature of 25° C. in sealed containers, regardless of whether the original storage time was 3, 6, 12, or 24 months. This was to be expected since that was the most unfavorable original storage condition used (Table I). These seeds germinated 96, 91, and 93 per cent respectively after 3, 6, and 12 months original storage and were indistinguishable in this regard from the seeds stored under all the other conditions. The inferior quality showed up, however, in the decreased resistance of the seeds to further unfavorable conditions, in this case paper packets at 30° C. and 76 per cent relative humidity. This response was similar to that obtained previously (1, 2) and pointed out in the introduction to this paper.

Reducing the moisture content of onion seeds from 10.6 to 3.5 per cent before storing in sealed containers at 25° C. made possible the preservation of their germination vigor for somewhat longer periods upon removal to 30° C. and 76 per cent relative humidity. Some of these effects, together with a comparison of germination capacity in soil and on moist filter paper and of seed envelopes and tin cans for packeting, are shown in Figure 2.

Several facts are evident. One of these is the increased tolerance of seeds stored with a low moisture content to subsequent unfavorable storage; another is the efficacy of tin cans for satisfactory keeping after packeting; and still another is the poor performance of weak seeds in the soil as compared to moist filter paper. Again, it should be emphasized that

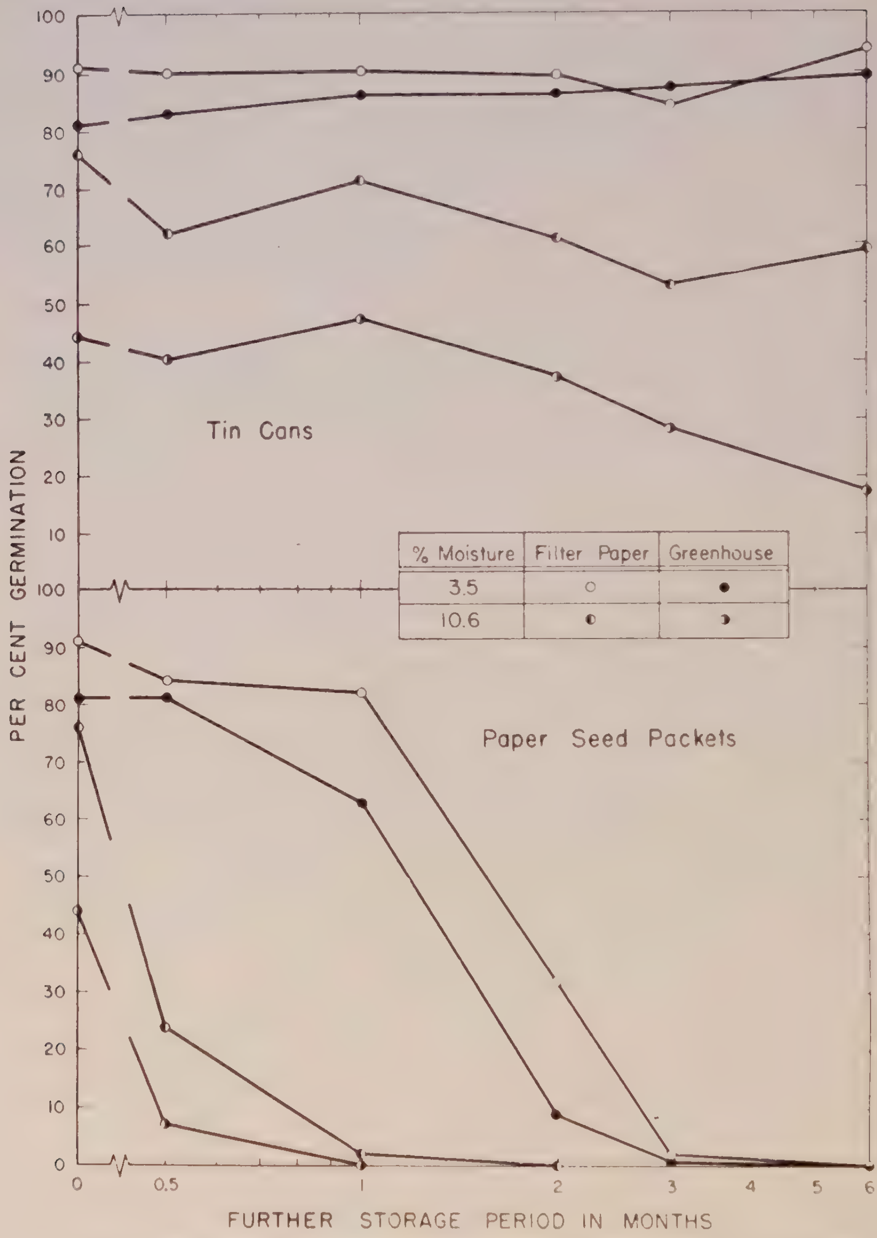

FIGURE 2. Germination on moist filter paper at 20° C. and in soil in the greenhouse of onion seeds with moisture contents of 10.6 and 3.5 per cent held for 24 months at 25° C. and then placed in paper seed packets or tin cans for a further storage period of one-half to six months at 30° C. and 76 per cent relative humidity.

only those seeds which had had previous dry storage and which had therefore been able to retain more of their initial germination energy, were able to survive six months of packeting, even when air-tight packets were used. That low temperature will bring about similar effects when the moisture content of the seeds is unfavorable is shown in Figure 3. Seeds with 10.6 per cent moisture stored for 6 or 24 months at 5° C. were much more tolerant of further storage in paper seed packets than those previously stored at 25° C. for the same length of time. This difference was especially striking at the 24-month period.

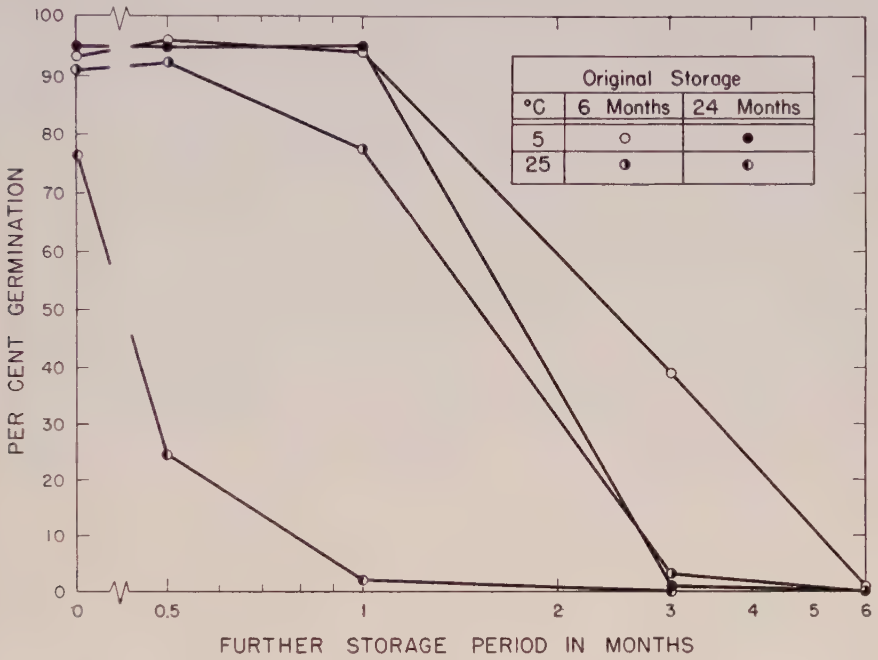

FIGURE 3. Germination on moist filter paper at 20° C. of onion seeds with 10.6 per cent moisture held at 25° or 5° C. for six and 24 months and then placed in paper seed packets for further storage period of one-half to six months at 30° C. and 76 per cent relative humidity.

SPECIAL SEED PACKETS[2]

From time to time, so-called moisture-proof plastic materials have appeared on the market. From these, water-resistant bags of various sizes have been made to protect such materials as food and textiles and parts of machinery of various kinds from the harmful action of excess moisture.

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[2] This part of the research was sponsored by Boyce Thompson Institute for Plant Research, Inc. after the termination of the W. Atlee Burpee Co. project.

Even though some moisture does penetrate the plastics, they have served the purpose of prolonging the usefulness of the encased materials in such places as the humid tropics.

Several of these new plastics have been tested to see whether they would protect seeds from excessive moisture. The ones tried in this laboratory have proved ineffective, and are as follows:

1. One-mm. moisture-resistant polyethylene cast film.
2. Two-mm. moisture-resistant polyethylene cast film.
3. Black plasticized vinyl film, .009″.
4. Extruded polyethylene film, .020″.
5. Calendered vinyl film, highly plasticized, .040″.
6. Calendered vinyl film with average plasticizer content.
7. Cast vinyl film with low plasticizer content.
8. Polyethylene cast film.

Heat-sealing was used in making seed envelopes of these materials, which were supplied from Bakelite Corporation, New York, N. Y. Seed envelopes have also been made by sealing three types of aluminum foil with heat. Two of these appear promising for use as seed packets. They are Metalam, a foil material with a scrim backing, furnished by the Kennedy Car Liner and Bag Company Inc., New York, N. Y., and a vinyl laminated aluminum foil from Bakelite Corporation. Both of these materials formed effective moisture barriers as measured by the moisture content of onion seeds stored in them under high humidity conditions for as long as three months. Foil Rap 40, a foil with kraft paper backing, seems to be less effective than the other two foil materials when the storage time is extended beyond three months. This may be due to the fact that kraft paper backing holds a layer of water next to the foil and thus more moisture gets through to the seeds. It should be recognized that there is some transmission of water vapor through all of these materials, and seeds stored in them will take up moisture in time. The moisture contents of onion seeds stored in bags made of three different plastics and three foils as well as the moisture absorbed by cucumber, pepper and tomato seeds in two types of plastic bags, are shown in Table II. Polyethylene cast film and cast vinyl film with low plasticizer content allowed moisture to reach the seeds, so that after one week at 76 per cent relative humidity there was a significant increase in the amount of water in the seeds. This was not true for the foil wrappers. Extension of the time in the humidity chambers to three months found the onion seeds protected by Metalam and vinyl-laminated foil with 6.3 and 6.6 per cent moisture. In another test, the moisture content of the seeds in the last two types of packets did not exceed 12 per cent in 64 weeks in spite of the 100 per cent relative humidity of the chamber in which they were enclosed. This severe test would seldom, if ever, be met in the storage of packets in this country. Certainly, when compared with regulation seed envelopes in which seeds attained a moisture content of 31 per cent in four weeks under the same conditions, the foil envelopes are far superior. A germination test of the onion seeds showed none viable after 8 weeks in the paper packets and 83 per cent still viable after 32 weeks in the foil envelopes. However, none of the seeds were viable after 64 weeks. The temperature in this case was 30° C.

TABLE II

MOISTURE CONTENTS OF SEEDS IN DIFFERENT TYPES OF PACKETS UNDER CONDITIONS OF HIGH HUMIDITY. MOISTURE PERCENTAGES CALCULATED ON BASIS OF DRY WEIGHTS OF SEEDS

Temp. and humidity	Packet material	Seed	Moisture in seeds after storage for weeks, %			
			0	1	2	3
30° C., 76% relative humidity	Polyethylene (#8)	Cucumber	5.5	7.7	8.2	—
		Onion	6.5	9.4	10.9	—
		Pepper	5.3	8.1	8.9	—
		Tomato	6.1	9.3	10.0	—
	Cast vinyl film (#7)	Cucumber	5.5	8.7	8.8	—
		Onion	6.5	11.7	11.8	—
		Pepper	5.3	9.2	9.6	—
		Tomato	6.1	10.5	10.3	—
	Foil Rap 40	Onion	5.7	—	5.9	—
		"	6.4	6.3	—	6.0
	Metalam	"	5.7	—	5.8	—
		"	6.4	6.7	6.4	6.1
Laboratory, over water	Foil Rap 40	Onion	6.6	6.7	—	6.6
	Metalam	"	6.6	6.6	—	6.7
	Vinyl-laminated aluminum foil	"	6.6	6.6	—	6.6
	Plastic tobacco pouch	"	6.6	14.2	—	24.4

The importance of perfect sealing of the foil envelopes for safety in seed storage cannot be overemphasized. If humid air is allowed to enter through an imperfect seal, the seeds acquire water readily and deterioration is hastened.

DISCUSSION

Some idea of the importance of the seed packet industry and of the poor quality of seeds found in many such packets may be gained from a series of articles by Munn and his co-workers, published between 1924 and 1949, two of which are cited at the end of this paper (5, 6). In 1926, 1927, and 1928, packets of vegetable seeds were purchased from 47 different seedsmen or dealers in New York. Out of 964 packets, 9.2 per cent were worthless for planting purposes. In addition to these, there was a relatively high percentage of packets of very little seeding value. In 1923, 23 per cent of 347 packets, and in 1924, 18 per cent of 600 packets were worthless. This represented very serious losses to the growers. The analysis, by these workers, of the germination behavior of the seeds by firms, with an actual listing of the firms and the quality of seeds they sold, has doubtless contributed to the forcing of some unscrupulous dealers out of the market.

An analogous situation has been found for flower seeds in New York (6). In 1936 of 500 packets of flower seeds examined, 57 per cent were excellent, good or entirely satisfactory, while the remaining 43 per cent were unsatisfactory. Fifteen per cent were worthless. Similar work has been reported from the Massachusetts Agricultural Experiment Station (7) and from the Seed Laboratory of the Department of Agriculture, Canada (4).

As a result of these and probably many other unreported tests, there are now Federal and State seed laws which seek to govern the type of seed which is offered for sale. From the fundamental seed behavior described in the present paper, however, it is evident that the problem of control is dependent upon a basic knowledge of the response of seeds to storage conditions, not only in the special storage room of the responsible seedsman, but also from the time they leave that room until they reach the grower.

Among the published reports on the germination of packeted seeds is that of Brown and Goss (3). Over a period of five years (from 1907 to 1911) they tested 18,571 packets of vegetable seeds. These were the familiar paper packets lithographed in colors and put up by sixty different firms. These paper packets are shipped in boxes and hence are known as box seeds. Mail-order seeds from twenty firms were also included in the study. A marked difference in the quality of the box and mail-order seeds was noted. Whereas the average germination of all of the former was 60.5 per cent, that of the latter was 77.5 per cent. When the germination of box seeds by firms was analyzed, the difference in average germination for the different firms was marked (from 81.5 to 36.5 per cent), while the difference in average germination of seeds from mail-order houses was small (from 81.8 to 70.3 per cent). Differences in box and mail-order seeds of the same variety ranged from 2.3 per cent for parsnip and 3.3 per cent for spinach, to 28.5 per cent for cabbage and 29.5 per cent for onion. The average germination of box seeds put out by any one firm was uniform from year to year, but the differences in quality varied from 73.7 per cent for one firm to 42.8 per cent for another. Many instances were found in which there were statements of high germination on the packet and yet the authors obtained 42.8 to 43.6 per cent germination. They concluded (3, p. 9), "It would thus appear that such firms either do not test their seeds accurately or else disregard the results of such tests to the extent of including lots showing unsatisfactory germination in their output of packeted seeds for the box trade." The dependence of mail-order firms on satisfied customers was given by Brown and Goss as the reason for the superiority of their seeds.

In the light of the evidence presented in the present paper, the better quality of the mail-order seeds might be attributed, at least in part, to the direct route from the favorable storage rooms of the seedsmen to the consumer. Such seeds would be subjected to possible high heat and humidity for a minimum period of time and hence have a higher germination capacity at planting time. Also, it may very well be that seeds which may have given 80 to 100 per cent germination at the time they were packeted for distribution lost their value entirely before they reached the grower, if the wholesale or retail establishment did not have good storage facilities. While this fact may remove some of the blame for seed failure from the seedsman, it is still his responsibility to provide the best possible seeds for filling the packets he sends out. This requires some combination of temperature and humidity which will maintain full germination vigor up to the time of packeting.

Also, the data presented here offer promise of a practical, moisture-resistant envelope of plastic-coated aluminum foil, which will not only increase the attractiveness of the seed packets, but which will give protection from rapid deterioration under adverse storage conditions.

SUMMARY

The effect of previous conditions of storage on the germination capacity of onion seeds after packeting has been determined. It was found that low moisture content (3.5 per cent of the dry weight of the seeds) and low temperature (5° C.) during storage increased the resistance of the seeds to harmful conditions following packeting for sale.

Tin cans, which provide air-tight packets, were far superior to kraft paper packets for maintaining the viability of onion seeds upon removal from storage rooms. The seeds kept well for six months to a year in tin cans at 30° C. and 76 per cent relative humidity, whereas in paper packets under the same conditions they were all reduced in vigor in three months so that good seedling stands could not be obtained in soil, and in six months they were all worthless.

Two metal foils, Metalam with a scrim backing, and vinyl-laminated aluminum foil, have been found to be satisfactory for moisture-resistant packeting of seeds and are described.

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TRIIODOBENZOIC ACID INFLUENCES FLOWER FORMATION OF TOMATOES

P. W. Zimmerman and A. E. Hitchcock

In 1942 Zimmerman and Hitchcock (10) reported that the pattern of leaves, the flowering habit, and correlation of organs were modified by means of 2,3,5-triiodobenzoic acid (TIB). They showed that flower clusters of tomatoes were induced to grow from axillary buds where leafy shoots normally appear and that terminal buds were replaced with flower clusters. The usual flower clusters also developed along the stem. The treated plants lost much of their apical dominance and produced an abnormally large number of axillary leafy branches. It was further pointed out that though benzoic acid was physiologically inactive, the molecule could be activated by the substitution of amino groups or halogen atoms in the ring (4, 7, 8, 9). The positions 2, 3, and 5 of the nucleus appeared to be the most important for substitutions. None of the monosubstituted compounds tested were found active. 2,5-Diiodobenzoic acid was active for modification and pattern of leaves but did not induce axillary flowers. TIB induced formative effects and also modified the position of flower clusters. 2-Amino-3,5-diiodobenzoic acid was inactive, but 2-chloro-3,5-diiodobenzoic acid was very active resembling TIB (8). The molecular configuration as a whole rather than any part of the molecule appeared to determine physiological activity.

It was further pointed out that the flower clusters of the Bonny Best variety of tomato (*Lycopersicon esculentum* Mill.) used in the experiments normally had 3 to 7 flowers per cluster but that under the influence of TIB some clusters formed as many as 35 flowers. Plants treated with 2-chloro-3,5-diiodobenzoic acid (11) produced flowers with very small, abnormal petals and long peduncles.

Galston (2) attempted to initiate flowering of soybeans by the use of TIB. Though he succeeded in increasing the number of flowers per plant from 32 on the control to 181 on the treated plant, Galston states that TIB does not possess florigenetic properties since it will not induce vegetative soybean plants to flower. He further stated that TIB caused morphological responses such as shortening of the internodes, loss of apical dominance, epinasty of young leaves, etc. but that the chemical itself was without auxin activity. The latter conclusion perhaps was drawn from the fact that TIB is not active on the *Avena* coleoptile.

Tumanov and Lizandr (5) found that TIB retarded growth of *Perilla* and caused formative effects. Alfalfa was more sensitive than *Perilla*. These authors found a difference in sensitivity during short and long

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days. The treatment caused a variation in the number and size of leaflets in alfalfa. Spraying with weaker solutions, 0.005 per cent, in long days brought about increased yield of alfalfa seed. Flax and sunflower species showed very notable changes in growth when treated with 0.01 per cent solution. This concentration also caused peas to branch through stimulation of axial buds and fusing of leaflets. There was, however, no definite sign that TIB could be considered as having florigenetic properties.

Owen (3) treated a number of species with TIB and brought about distortions of leaves, stems, and flowers but failed to change any organ from the vegetative to the flowering stage.

Avery *et al.* (1) say that the induction of flowers with TIB in place of vegetative tissue in tomato strongly suggests that the substance plays the role of flower-inducing hormones in some plants.

The best support for the earlier findings of Zimmerman and Hitchcock was published by Waard and Roodenburg (6). They state that TIB is the only synthetic substance known to influence formation of flowers. These workers found that the treatment with TIB caused the flower cluster to develop at the top of the plant while the vegetative shoot was suppressed. This was considered as a shifting of the correlative relations between the vegetative shoot and flower cluster. They also reported an increase in the number of initiated flower buds and the formation of axillary flower buds when the plants had very few leaves. In fact the illustration shows the flower buds arising from plants with only cotyledon leaves. They concluded that the chemical has the property of starting the process of flower formation and also that it is possible to shorten the vegetative juvenile stage of tomatoes.

The purpose of the present report is to show that the earlier published work has been repeatedly verified by additional experiments, and further to record some of the more recent findings.

METHODS AND MATERIALS

The principal methods which have been used for treating plants with TIB are as follows: addition of the chemical directly to the soil in which the plant grows; lanolin preparation containing the chemical applied to the aerial parts; and water solutions sprayed on the leaves. The concentration range when applied to sandy loam soil was 0.2 mg. to 10 mg. per 50 ml. of water. Only one application to each pot was necessary, and all of the solution was applied at one time. Lanolin preparations of concentrations ranging from 1 to 10 mg. per g. of lanolin were applied around the stem or at local places but usually near the uppermost portion of the stem. The plants varied from 2 to 6 inches in height. Water solutions containing 100 to 500 mg. per liter of water were sprayed on the plants by means of an atomizer. The spray treatment was often repeated several times. Other methods which are very effective but have not been much used are injection of the solution with a pointed glass capillary tube, and the use of a wick with one end in the solution and the other wrapped around the stem.

EXPERIMENTAL RESULTS

INDUCTION OF AXILLARY AND TERMINAL FLOWER CLUSTERS OF TOMATOES

Tests were conducted during the summer months and again during the winter. There was a direct relationship between vigor of the plants or rate of growth and the effects of the chemical. During the long days when plants grew rapidly, axillary flower clusters could be induced with as little as 0.2 mg. of TIB dissolved in 50 ml. of water and applied to the soil of a four-inch pot. Ten milligrams per pot strongly inhibited most of the tomato plants during the summer tests. During the winter the plants grew slowly, and, though they were stunted, they did not produce axillary flower clusters with a range of concentrations from 0.2 to 5 mg. per pot of soil. In another experiment started on December 12 but with six hours of artificial light (from 1000-watt nitrogen filled bulb) to supplement daylight, the experimental plants produced both axillary and terminal flower clusters as well as clusters along the stem between the nodes.

The number of axillary flower clusters per plant and the number of terminal flower clusters (in place of a vegetative growing point) are tabulated in Table I. The plants referred to in the table were treated December 22 and the flower count was made February 23. It can be seen from these data that the chemical caused an abnormally large number of flowers per cluster and per plant. For example, the controls during this experiment usually had 3 to 5 flowers per cluster and a total of 10 to 15 flowers per plant. The average for all treated plants was 4 axillary clusters with a range of 1 to 32 flowers per cluster. Terminal and internodal clusters also averaged more than the normal number of flowers.

A typical response of a plant treated by way of the roots with 4 mg. applied to the soil of a four-inch pot is shown in Figure 1 A. Beginning at the top, the picture shows what is assumed to be a flower cluster replacing the terminal growing point; an internodal, branched flower cluster; and five axillary clusters. At least one axillary cluster was mixed with leaves but did not have a growing point. The terminal cluster often developed merged or fasciated flowers surrounded by a number of small buds. Some but not all of the surrounding buds matured. The fasciated ones in the picture produced a tubular pistil with a fringe of stigmatic surface (Fig. 1 B and C). When pollinated, a circular ovary developed. In at least one case a circular ovary developed at the top of a tubular peduncle. Sepals surrounded the ovary and also protruded from the inside of the tube. Small buds also developed inside the tube. There were many variations of

TABLE I

INFLUENCE OF 2,3,5-TRIIODOBENZOIC ACID ON THE NUMBER OF FLOWERS AND POSITION OF FLOWER CLUSTERS

Treatment mg./pot or mg./g. lanolin	No. of clusters		No. of flowers			Remarks
	Terminal	Axillary	Per cluster	Total	Av.	
TIB added to soil						
10	1	8	7, 10, 5, 6, 8, 12, 1, 25, 32	106	12	Many peduncles branched.
7.5	0	3	1, 6, 16	23	8	Many small undeveloped flowers. Some abnormally long peduncles with fasciated flowers.
5	1	5	8, 10, 25, 4, 10, 9	66	11	Many branches on plant. Peduncles branched and long. Both internodal and axillary flowers modified.
4	1	5	6, 4, 5, 16, 1, 10	42	7	Fasciated flowers with tubular pistils which set fruit. Calyx often failed to separate.
4	1	2	2, 25, 8	35	12	Many small flowers. Abnormally long peduncle with node-like structures.
3	1	4	1, 2, 6, 15, 6	30	6	Abnormal branching of peduncles, and long axillary leafless shoots.
3	1	3	1, 5, 17, 20	43	11	Abnormal branching of peduncles, and long axillary leafless shoots.
1	1	2	8, 18, 7	33	11	Abnormally long axillary shoots with leaves only at tip.
TIB applied as lanolin						
10	0	4	5, 1, 6, 18	30	8	Practically all axillary buds with growth.
7.5	0	3	8, 5, 6	19	6	Practically all axillary buds with growth.
5	0	3	8, 5, 12	25	13	
Control	0	0				Plant had two internodal flower clusters with 3 and 5 flowers.
Control	0	0				Plant had two internodal flower clusters with 3 and 5 flowers.
Total and average				452	9	

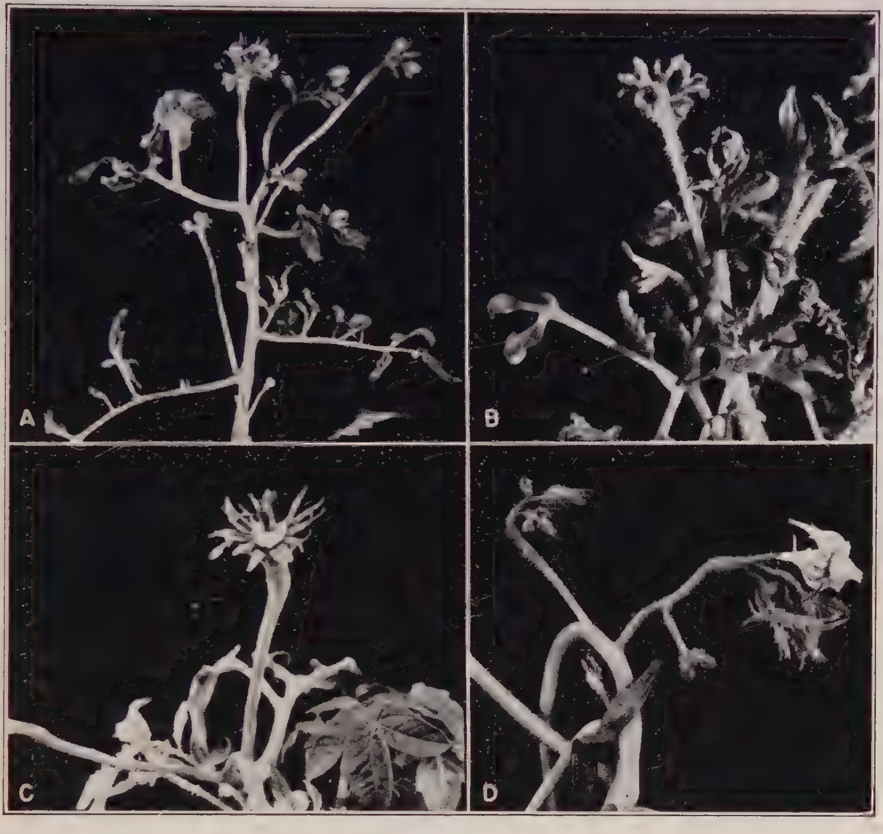

FIGURE 1. Tomato plants. A. Uppermost portion of a plant showing axillary flower clusters developed from vegetative tissue and terminal flower cluster supplanting the vegetative growing point. The plant was treated on December 22 with 4 mg. of TIB in 50 ml. of water applied to the soil. Photograph was taken the following February 16. B. Uppermost portion of a tomato plant treated with a lanolin preparation of TIB 10 mg./g. of lanolin on December 22. Photograph was taken February 16. The picture shows axillary flower cluster developed from vegetative tissue. The cluster has one large circular flower and an abnormally large number of associated smaller buds. C. Tomato cluster. Tubular peduncle with circular ovary induced with TIB 10 mg./50 cc. of water applied to soil January 11. Photograph was taken on March 8. Note sepals within the circular ovary which developed after pollination. D. Terminal and internodal flower cluster induced with 10 mg. TIB/50 cc. of water applied to pot January 11. Photograph was taken March 8. Internodal peduncle is branched and has one abnormally large flower.

this which are difficult to describe. Peduncles were often abnormally long and thick with enlargements resembling nodes. These unusual peduncles had stem-like structures, but were not unlike large peduncles of normal plants.

Axillary flower stalks varied from single stalks with no buds but with some color on the upper end to what appeared to be normal or very large cluster peduncles. The latter formation shows in Figure 1 A at the lowest axillary growth. Such flowers did not mature. This type of flower was induced with 2-chloro-3,5-diiodobenzoic acid and was referred to in an earlier publication (8, p. 112). Waard and Roodenburg (6) described and illustrated this type of flower on very young seedlings in a recent publication. Possibly the chemical initiates the flower primordia, but the plant has not produced enough supporting substances or food material to bring about full development.

Instead of forcing the plant to absorb the active substance through the roots, preparations made with TIB in lanolin were applied to aerial parts of the plant, usually around the stem near the tip. Figure 1 B shows an enlargement of a portion of a plant treated on December 22 and photographed the following February 16. The picture shows an axillary flower cluster with a large bud (perhaps several buds formed into a circle) and many small buds surrounding. The long, heavy peduncle is very prominent. After the picture was taken this large bud developed a circular pistil and something resembling petals. The flower was hand pollinated and developed a circular growth of the ovary with an open center. Some but not all of the associated buds developed into flowers and set fruit.

The change in number of flowers per cluster and the modification of floral parts occurred on clusters arising from internodal regions as well as terminal and axillary clusters. The fact that treated plants had both internodal and axillary clusters indicates that the latter were induced over and above those that normally occur.

Plants growing vigorously in June responded to lower concentrations of TIB than slow-growing plants in winter. More work needs to be done on this phase of the problem to determine the effects of short day, temperature, and a combination of these two factors.

During June axillary flower clusters were induced with concentrations ranging from 0.2 mg. to 10 mg. per 50 ml. of water applied at one time to the soil of a four-inch pot. When solutions were added to soil, considerable variation in response of what was thought to be comparable plants was evident. It was assumed that at least part of the variation could be accounted for by the fact that the soil was not physically homogeneous or uniformly moist. The response was also different with plants growing in sand and loam soil. Larger amounts of TIB were tolerated by plants in clay soil than in loam, and plants in loam tolerated more than plants in sand. Water culture has been used but not fully standardized.

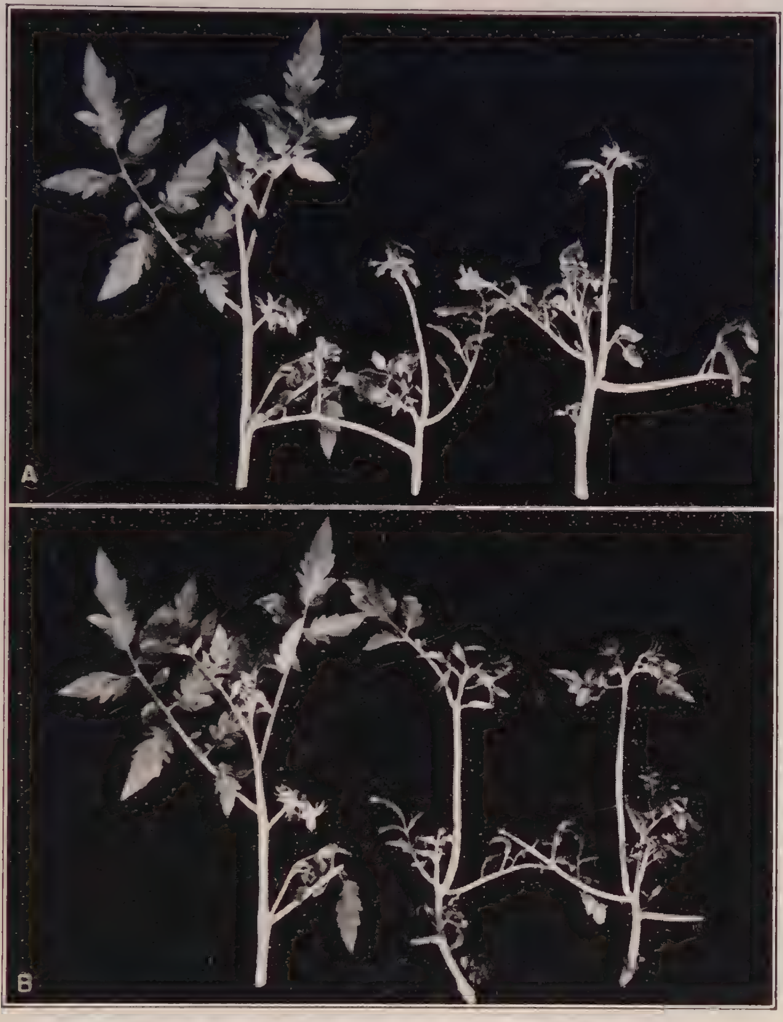

FIGURE 2. Tomato shoots showing effect of TIB. A. Tomato stems to show effect of applying 5 mg. of TIB in 50 ml. of water to soil of a four-inch pot on June 5. Photograph was taken July 17. Left, control stem with normal flower cluster; middle, axillary flower cluster with an abnormally long heavy peduncle; right, two axillary flower clusters, one with an abnormally large flower and long peduncles, and the other with a short peduncle and unorganized yellowish flower tissue at the tip. B. Three tomato shoots. Left, control with a normal flower cluster and axillary leafy shoot; middle, one large flower cluster having also two leaves and long peduncle with several node-like structures; right, axillary flower cluster developed from vegetative tissue. The flower cluster has also a few leaves. The flowers are abnormally large and peduncle abnormally long.

Plants growing in June under the influence of TIB also produced axillary flower clusters with long, heavy peduncles. These were without growing points for shoots, but in some cases leaves were mixed with flower buds. Two types are illustrated in Figure 2. Some of the axillary buds produced short peduncles with color at the tip, as illustrated in Figure 1 B. It was assumed that the tip was composed of unorganized cells which may lack the associated structures for normal flowers.

The enlargements around the abnormally long peduncles which are evident in the illustrations resemble nodes without associated leaves or buds. The structure is now being studied and compared with normal stem and peduncle structure. An examination of peduncles on large control plants disclosed that the node-like structures are not uncommon.

Spray solutions with concentrations ranging from 200 to 500 mg. per liter induced approximately the same responses as described for soil treatments and application of lanolin preparations. Repeated sprayings (two or more) of low concentrations (50 to 150 mg. per l.) were as effective as one spraying with 200 mg. per l.

DISCUSSION

Attention was called in previous publications (4, 8) to the fact that the 2, 3, and 5 positions in the benzene ring appeared to be most important to make an active molecule in the benzoic acid series. In contrast to the substituted phenoxy acids, the para position is not an important location in the molecular configuration for active derivatives of benzoic acid. Not all possible substitutions have been made, but from the information at hand the halogen substitutions in the 2, 3, and 5 positions make the molecule more active than the substitution of amino or nitro groups. For example, 2-amino-3,5-dichlorobenzoic acid is inactive while 2-chloro-3,5-diiodobenzoic acid is very active for modification of leaves and induction of axillary flower clusters. 2,5-Dichlorobenzoic acid is especially active for inducing parthenocarpic fruit and modified leaves but does not induce axillary or terminal flower clusters.

Since the effects of TIB vary in summer and winter with the rate of growth of tomato plants, it is evident that the substance cannot work alone. It must depend upon materials made by the plant in order to cause vegetative tissue to produce flowers. It is assumed that plant hormones also require supporting substances produced by the plant to cause well-known physiological responses. The rooting response after treatment with indolebutyric acid is conditioned by the age of the experimental species, the environmental growing conditions, and the storage of material in the treated tissue. From this angle it appears that TIB and 2-chloro-3,5-diiodobenzoic acid may be considered to have hormone characteristics.

SUMMARY

The review of literature shows that other workers have given support to reports in earlier publications that 2,3,5-triiodobenzoic acid (TIB) increases the number of flowers on treated plants and causes flowers to develop from vegetative tissue. Repeated experiments with tomato plants prove that TIB causes axillary buds to develop flower clusters instead of leafy shoots. Branches and main tomato shoots were caused to terminate in flower clusters appearing to supplant a shoot growing point. Treated tomato plants also developed flower clusters in the usual places along the stem, but they frequently had fewer or more than the normal number of flower buds. In fact, internodal, axillary, and terminal flower clusters were similar and were characterized by extremes from short to long, heavy peduncles and fasciated flowers mixed with small buds. Upon recovering from the chemical influence plants often produced abnormally large flowers. Some of the fasciated flowers when pollinated produced a circular ovary resembling a doughnut. Such ovaries had sepals both within and around the ovary. Under certain conditions the axillary peduncles had only yellowish cells at the tip, indicating unorganized flower cells.

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WATER HYACINTH: ITS GROWTH, REPRODUCTION, AND PRACTICAL CONTROL BY 2,4-D[1]

A. E. HITCHCOCK, P. W. ZIMMERMAN, HENRY KIRKPATRICK, JR., AND T. T. EARLE[2]

Water hyacinth (*Eichhornia crassipes* Solms.) has been recognized as a serious pest in waterways of the Gulf Coastal area of the United States for more than 50 years (3, 11, 13, 14) and also in Ceylon (6). Estimates of the damage which this species causes annually range from 5 to 14 millions of dollars (3, 11). Hundreds of thousands of dollars are reported to have been spent annually during the last 40 years merely to keep open channels through the dense masses of water hyacinth (13). Although a number of attempts have been made to control these plants by means of 2,4-D (2,4-dichlorophenoxyacetic acid) and other chemicals, there are relatively few published reports on this subject. Of several reports relating to the use of 2,4-D in controlling water hyacinths prior to 1948 (2, 3, 7), only the one by Hildebrand (7) gives any substantial and detailed information concerning concentration of 2,4-D and its rate of application and delivery.

According to Hildebrand (7) hyacinths in Florida were killed in most cases in two to three weeks by one to three applications of 0.1 per cent 2,4-D applied at the rate of 1 gal. per 100 to 150 sq. ft. The maximum concentration of 2,4-D was 0.125 per cent and the maximum rate applied was about 3.3 lb. per acre. Five different formulations of 2,4-D (the acid, two salts, and two esters) were of equivalent activity. The use of Carbowax 1500 as a spreader did not increase the activity of 2,4-D. Experimental water hyacinths were located in a stream having a noticeable current, in relatively quiet water, and in drums of 50 gal. capacity. The final fate of treated hyacinths was determined with certainty only for those in drums, in which case all plants died, decomposed, or settled to the bottom. The doses of 2,4-D used by Hildebrand (7) were considerably lower and the rate of killing and sinking was two to four times as rapid as compared with the present results in Louisiana during the period May, 1948, to February, 1949. A preliminary report of the results obtained in Louisiana was made in January, 1949 (8). The present report gives additional and more detailed information relating to the results of field work than was

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[1] These investigations were carried out by members of the staffs of Tulane University, New Orleans, Louisiana, Boyce Thompson Institute for Plant Research, Inc., and the U. S. Corps of Engineers, New Orleans District. The project was made possible by a contract between Tulane University and the Corps of Engineers and had the cooperation of Colonel John R. Hardin, District Engineer, and his staff.

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contained in the first report (8) and, in addition, summarizes the results of laboratory and greenhouse tests.

Field work dealing with the eradication of water plants in Louisiana was started in April, 1948. The work involved laboratory and field studies relating particularly to the growth and reproductive habits of water hyacinth and alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb.) and their practical control by treatment with chemicals. Since control of water hyacinth involves both killing and sinking, these two phases are discussed under separate headings.

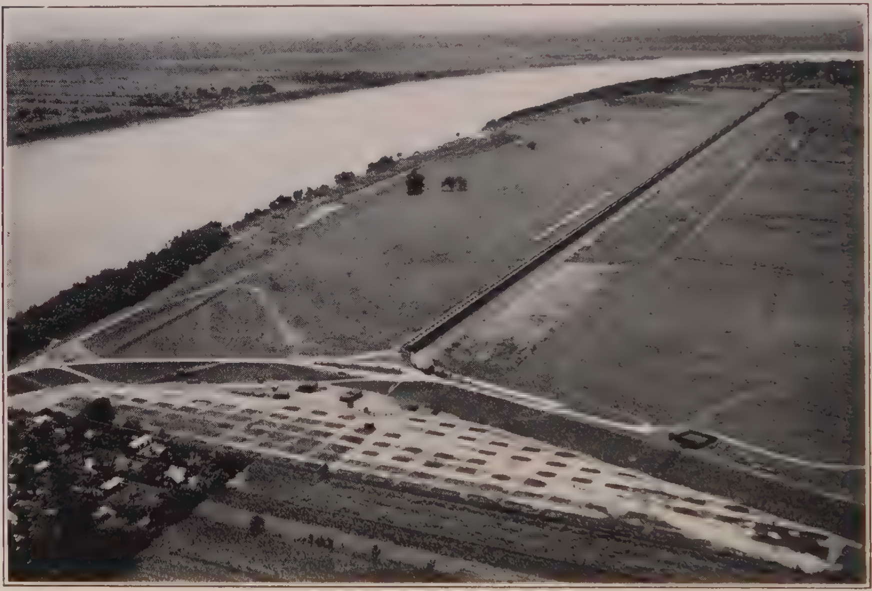

FIGURE 1. View of experimental pits adjacent to Bonnet Carré Spillway, Norco, La.

METHODS OF PROCEDURE

Field plots. The principal treatments were applied to plants growing in 108 pits located at Norco, Louisiana, in the Bonnet Carré Spillway area (Fig. 1). Each pit was approximately 15×30 ft. at the top, 10 ft. deep, and 10×10 ft. at the bottom. During dry periods the water level was maintained at one to two feet below ground level depending upon the size of the plants. In addition to rain water, the pits contained natural seepage and water pumped from the Mississippi River. The growth and appearance of the water hyacinths (hereafter referred to as hyacinths) were similar to those of plants growing under natural conditions.

Hyacinths growing under natural conditions in canals or borrow pits served also for experimental work. A borrow pit refers to any excavated area which contains water. Areas of about 50 sq. ft. were sprayed with a Hudson Junior sprayer having the same type of nozzles as those used for spraying plants in the pits.

Equipment and materials. Most of the test solutions were applied to plants in the pits at a pressure of 25 to 30 lb. at rates of 6 to 150 gal. per acre by means of a four-nozzle boom which delivered from each nozzle a flat fan-shaped spray pattern. The calculated concentrations of 2,4-D required for different rates of application for each of the five Spraying Systems nozzles are shown in Table I. An area of about 100 sq. ft. at the ends of the pits was treated, the middle portion of the pit serving as a non-treated control. In some tests all plants in a pit were treated.

TABLE I

CALCULATED PERCENTAGE CONCENTRATIONS OF 2,4-D REQUIRED FOR DIFFERENT RATES OF APPLICATION FOR EACH OF FIVE SPRAYING SYSTEMS TEEJET NOZZLES

Lb./acre	Nozzle number and rate of delivery in gal./acre*				
	650067**	6502†	8006	8010	8020
	6	18	43	57	70
2	3.90	1.36	0.56	0.42	0.34
4	7.80	2.72	1.12	0.84	0.68
8	15.60	5.44	2.24	1.68	1.36
16	31.20	10.88	4.48	3.35	2.72

* Rate of delivery (gal./acre) based on a pressure of 25 lb. p.s.i., a speed of 3 m.p.h., a nozzle spacing of 20 in., and a spray height of 19 in.

** With 100-mesh screen.

† With 50-mesh screen.

The low volume, low pressure spraying equipment, consisting of a boom, a gasoline-powered compressor, and a storage tank with a reducing valve, was mounted on a rubber-tired wheelbarrow which facilitated moving from place to place (Fig. 2). Treatments applied by means of this equipment are listed in Table II. In addition, there were 247 tests in which individual hyacinths were sprayed with a DeVilbiss No. 114 atomizer or the Hudson Junior sprayer. A Bean gun sprayer operated at 400 lb. pressure was also used for applying 2,4-D sprays to hyacinths growing in borrow pits.

An efficient spreader is considered essential for obtaining good coverage on the dense smooth-surfaced foliage of hyacinths. Results obtained with 1 per cent solutions of spreaders, detergents, and solubilizers (Table III) show that six of the seven were equally effective. Since Nekal NS (General Dyestuff Corp.) proved to be an effective spreader when used at a concentration of 0.25 per cent, it was used in most of the tests. Most sprays were

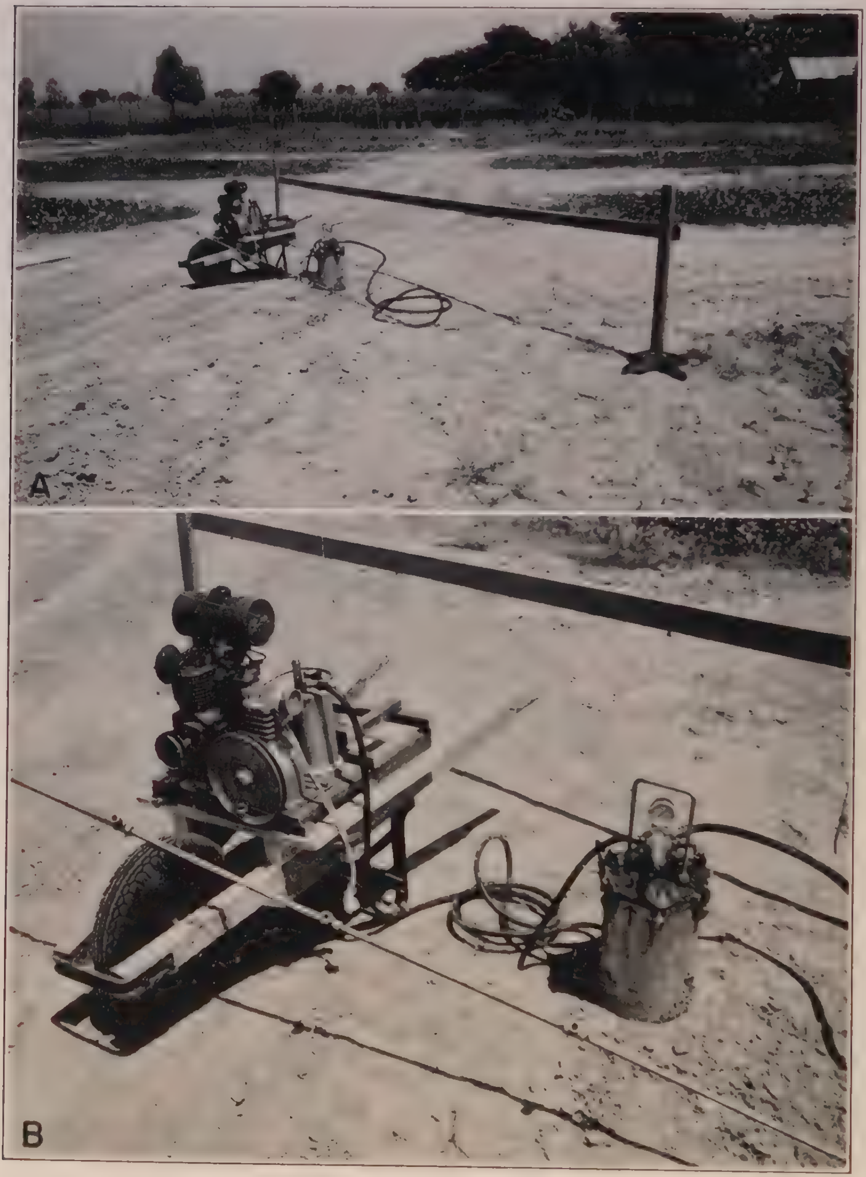

FIGURE 2. A. Portable spray equipment used for spraying plants in experimental pits. The adjustable bar with plumb lines was set at a predetermined height and was then used as a runway for one end of the four-nozzle boom. B. Close-up of equipment, shown above, consisting of a gasoline powered compressor, storage tank with reducing valve, four-nozzle boom with connecting hose, guide bar, and wheelbarrow.

TABLE II

NUMBER OF TREATMENTS APPLIED TO PLANTS GROWING IN PITS

Principal spray ingredients	Hyacinth		Alligator weed		Totals
	Ends of pits	Entire pits	Ends of pits	Entire pits	
Alkanolamine salt of 2,4-D	67	11	7	—	85
Sodium salt (different spreaders)	8	—	—	—	8
Esters of 2,4-D	11	—	7	—	18
Alkanolamine salt of 2,4,5-T*	7	—	4	1	12
Esters of 2,4,5-T	3	—	4	1	8
Non-hormone toxicants	9	1	2	—	12
Mixtures (hormone + toxicant)**	15	1	7	—	23
Successive (hormone, toxicant)**	6	—	—	—	6
Mixtures (two hormone types)	7	—	1	—	8
Total number of pit treatments	133	13	32	2	180

* 2,4,5-Trichlorophenoxyacetic acid.

** The term toxicant used alone refers to a non-hormone type of chemical.

applied when the foliage was not noticeably wet and when it appeared unlikely that rain would fall within two hours after treatment. However, the effectiveness of a 2,4-D treatment was not reduced when the sprays were applied to wet hyacinth foliage, but a marked reduction occurred if it rained within two hours after the spray was applied. In some cases, particularly with the low volume nozzles, it appeared that the sprays were more effective if applied to foliage which was wet as a result of a previous rain or dew.

TABLE III

RELATIVE EFFECTIVENESS OF DIFFERENT ADJUVANTS USED AT A CONCENTRATION OF 1 PER CENT IN A 1.2 PER CENT SOLUTION OF 2,4-D SODIUM SALT APPLIED TO HYACINTHS AT THE RATE OF 8 LB./ACRE ON AUGUST 9, 1948

Spreader	Percentage top kill		No. plants not killed in 36 days
	After 21 days	After 36 days	
None	80	99	4
Tween #20	95	100	0
Nekal NS	98	100	0
Tergitol #7	95	99	3
Corikal B	90	100	0
Igepon T	95	100	0
Igepal 300	98	100	0
Igepon AP	99	100	0

The amine salt of 2,4-D refers to the formulation of 2,4-Dow Weed Killer containing the equivalent of 40 per cent of the free acid in the form of the alkanolamine salt. This formulation does not specify the particular amine salts used. Likewise, the alkanolamine formulation of 2,4,5-T (2,4,5-

trichlorophenoxyacetic acid) did not specify which amine salt or salts were used. All 2,4-D and 2,4,5-T spray solutions were made up on the basis of the free acid equivalent regardless of whether the concentrates were salts or esters.

Screening tests. In addition to the compounds listed in Table X, the following substances were used alone and in combination with 2,4-D in preliminary screening tests: diallyl maleate, ammonium thiocyanate, sodium chloride, trichlorobenzene, *o*-dichlorobenzene, and ammonium sulfamate. A sticker, Geon 31X, and the spreaders listed in Table III were also used with different formulations of 2,4-D. Screening tests consisted of spraying individual hyacinths in duplicate with a DeVilbiss No. 114 atomizer or with a Hudson Junior sprayer. None of the non-hormone compounds was as effective as the salt and ester formulations of 2,4-D.

Treatment of individual hyacinths. The amine salt of 2,4-D caused quicker killing of individual plants as compared with the results obtained with similar 2,4-D sprays applied to undisturbed hyacinths in the experimental pits and in borrow pits. For example, a 1 per cent solution of 2,4-D killed individually treated hyacinths in 7 days whereas those treated in the experimental pits were seldom killed in less than 21 days. When one to four leaf blades on hyacinths were treated with 1 per cent 2,4-D, the plants died in 22 days as compared with 7 days when all leaf blades were sprayed. Presumably all leaves on a hyacinth do not have to be sprayed with 1 per cent 2,4-D in order to cause complete killing of the entire plant. The importance of the concentration of 2,4-D was evidenced by the fact that more than one-fourth of the foliage of hyacinths had to be sprayed with a 0.1 per cent solution of 2,4-D in order to cause killing of the entire plant.

Spraying a parent hyacinth plant with 0.1 to 1.0 per cent of the amine salt of 2,4-D killed the plant but did not kill the attached offshoots, although typical hormone responses and some injury occurred on the daughter plants when the 1 per cent solution was used. Similar results were obtained in greenhouse tests. Penfound and Minyard (12) also reported little transport of 2,4-D from parent to offshoots. When one of two attached offshoots was forced to grow in the air and consequently did not develop a root system in water, the offshoot in air died, but the one with roots in water did not die after treatment of the parent plant with a lethal dose of 2,4-D (1 per cent). The same results were obtained when 2,4-D was added to the solution in which the parent plant was growing. In this case the attached offshoots with well-developed root systems were grown in separate containers. These results show that there is relatively little transport of 2,4-D from a treated hyacinth plant through the stolon to an attached offshoot having a well-developed root system of its own. This is in contrast to the efficient transport of 2,4-D within a single hyacinth in which case killing of the entire plant occurred as a result of treating one or two leaves with 1 per cent 2,4-D.

Preliminary field tests. Hyacinths growing under natural conditions in a borrow pit were used for the preliminary testing of 2,4-D and other hormone herbicides in which case duplicate plots of 50 sq. ft. each were sprayed by means of a Hudson Junior sprayer. Results of these tests which were carried out during the month of May, 1948, showed that effective doses of 2,4-D were considerably higher than those reported by Hildebrand (7) for killing hyacinths, that the esters of 2,4-D were of about the same effectiveness as the amine salt, that 2,4-D formulations were more effective than their 2,4,5-T analogs, and that plants with roots in soil were more readily killed than plants with roots in water. Final evaluation with respect to killing of underwater parts of hyacinths and sinking of the plants was not possible due to the interference caused by the encroachment of adjacent non-treated plants into the treated area. Non-hormone toxicants could not be used because cattle had access to this particular area. This is in contrast to the results obtained in the experimental pits where the fate of the treated plants could be followed until they sank, and non-hormone toxicants could be used since the experimental pit area was completely surrounded by a cattle-proof fence.

KILLING HYACINTHS

2,4-D amine salt. Results of preliminary tests started in May, 1948, indicated that the amine salt of 2,4-D was effective in killing hyacinths and of nearly the same effectiveness as the isopropyl and butyl esters. Because of the known drift hazards associated with the use of 2,4-D esters, the amine salt was used in later tests to a much greater extent than ester formulations (Table II). Various stages of disintegration of hyacinths following treatment with 2,4-D are shown together with control plants in Figure 3.

The sensitivity of hyacinth leaves to 2,4-D increased with the age of the leaves, the older leaves being the first to discolor and die. The relative degree of browning within three days after treatment served as an approximate indication of the dose of 2,4-D which had been applied. An effective dose of 2,4-D, which eventually killed 90 per cent or more of the hyacinths, caused noticeable browning on the middle-aged and on some of the younger leaves within 48 hours. 2,4-D sprays containing 0.25 per cent Nekal NS caused a more uniform and more extensive browning of the leaves than a similar spray not containing Nekal NS or containing a less effective spreader, such as Tergitol #7 or Tween #20. Any 2,4-D treatment which failed to cause an additional and noticeable browning of hyacinth foliage within three days proved to be an ineffective treatment regardless of the time of year the spray was applied. When hyacinth leaves discolored or

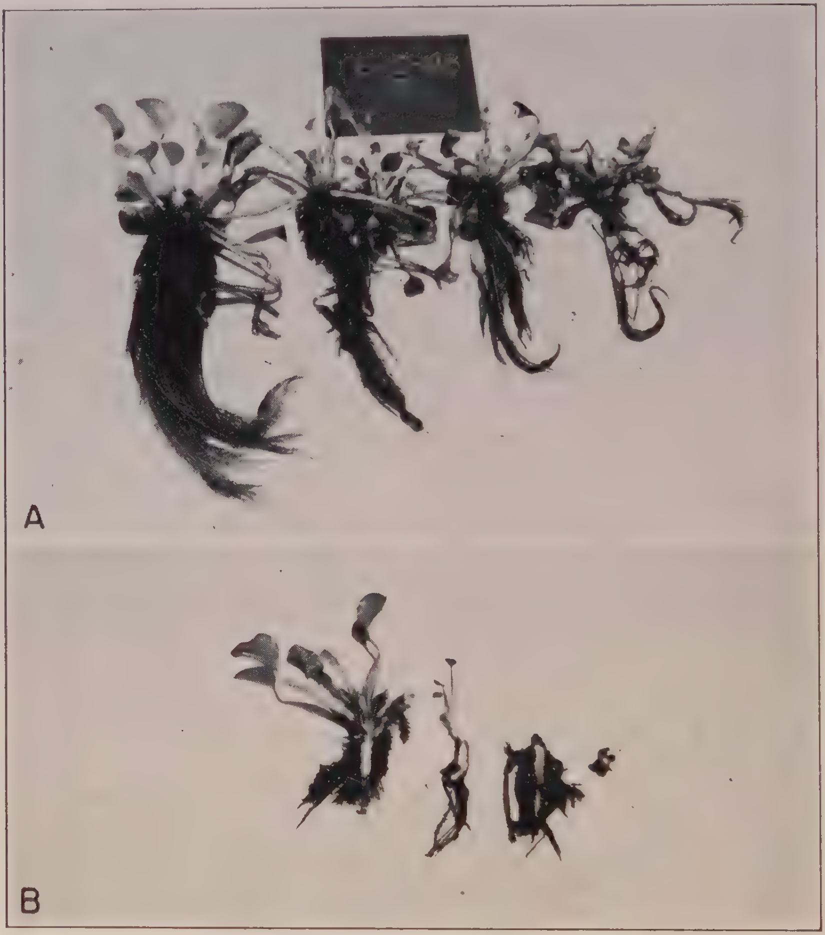

FIGURE 3. Various stages of disintegration of hyacinths treated with 2,4-D. A. Rhizomes uncut. B. Rhizomes cut longitudinally to show submerged tissue which if not killed is capable of producing new leaves after losing original ones as in case of second plant from left. Controls on left in A and B.

died as a result of being subjected to greatly reduced light intensity or to adverse weather conditions in the fall and winter seasons, the youngest leaves were the last to become affected as was the case when the plants received an effective dose of 2,4-D.

The amine salt of 2,4-D killed 90 to 100 per cent of the hyacinths growing in the experimental pits during the months of June to October inclusive, regardless of flowering cycles, when the concentration of 2,4-D was 0.3 per cent or higher, the rate of application 2 lb. per acre or higher, and the rate of delivery 6 to 150 gal. per acre. The principal limiting factors in killing hyacinths were concentration and rate of application of 2,4-D, rate of delivery of the spray solution, stage of growth and development of the hyacinth, and atmospheric conditions. Variable results were obtained with the two lowest volume nozzles, particularly with the 6 gal. per acre nozzle, presumably due to incomplete coverage. At these relatively low volume delivery rates spray drift was noticeable and varied with wind conditions.

Relationships between the percentage or number of hyacinths killed and concentration of 2,4-D, rate of application, and rate of delivery are shown in Tables IV, V, and VI. In general the effectiveness of 2,4-D increased with increasing concentration or rate of application as indicated in Figure 4, but there appeared to be no consistent relation to the rate of delivery. When the 62 treatments with the amine salt of 2,4-D applied during the period May to August inclusive were ranked according to the percentage of hyacinths killed, the rank difference correlation coefficients for concentration ($r' = .4408$) and rate of application ($r' = .4891$) approached significance ($r' = .553$ required) by Spearman's method (10, p. 472). In contrast, the r' value for percentage of plants killed according to rate of delivery was $r' = 0.0000$. Similar results were obtained by using Friedman's method for calculating the rank difference correlation coefficients (5). Failure to obtain significant rank difference correlation coeffi-

TABLE IV

RELATION BETWEEN CONCENTRATION OF 2,4-D AMINE SALT AND THE RATE OF APPLICATION AND DELIVERY FOR KILLING AND SINKING HYACINTHS GROWING IN PITS AS NOTED 67 DAYS AFTER TREATMENT (JUNE 24, 1948)

Concn., %	Gal./acre*	Calculated lb./acre	No. plants present**	% Plant material sunk	
				In each plot	Av. for group
0.16	70	0.9	3	25	33
0.30	57	1.4	1	25	
0.32	70	1.9	2	50	
0.52	43	1.8	14	85	73
0.60	57	2.8	0	50	
0.64	70	3.8	2	85	
1.04	43	3.7	3	90	93
1.20	57	5.6	0	98	
1.28	70	7.6	2	95	
1.38	18	2.0	0	90	
2.08	43	7.4	0	95	96
2.40	57	11.2	0	98	
2.76	18	4.0	8	95	

* Based on measured delivery at rate of 3 m.p.h. from 4-nozzle boom.

** Area treated was 100 sq. ft., containing approximately 2,000 plants.

TABLE V

RELATION BETWEEN CONCENTRATION OF 2,4-D AMINE SALT AND THE RATE OF APPLICATION AND DELIVERY FOR KILLING AND SINKING HYACINTHS GROWING IN PITS AS NOTED TWO MONTHS AFTER TREATMENT (AUG. 17, 1948)

Concn., %	Rate applied		% Plant material sunk*		No. new plants after 64 days
	Gal./acre	Lb./acre	After 55 days	After 64 days	
0.68	70	4	50	95	0
0.84	57	4	25	90	0
1.12	43	4	60	95	0
3.72	18	4	70	100	0
7.80	6	4	10	90	8
1.36	70	8	70	95	0
1.68	57	8	60	100	0
2.24	43	8	70	100	0
7.44	18	8	80	95	0
15.60	6	8	70	95	0

* The 6 gal./acre rate with 7.8 per cent 2,4-D caused 95 per cent kill and all other treatments caused 100 per cent kill.

TABLE VI

EFFECTIVENESS OF THE 2,4-D AMINE SALT 55 DAYS AFTER APPLYING (OCTOBER 5, 1948) TO ALL HYACINTHS IN A PIT

Concn., %	Gal./acre	Lb./acre	Plants killed, %	No. new plants
0.3	70	2	95	25
0.55	43	2	95	24
1.38	18	2	98	15
0.6	70	4	100	0
1.1	43	4	99	5
1.2	70	8	>99	1

cients is due to insufficient dispersion of the percentages of plants killed, only 4 of the 62 values being less than 90 per cent. As will be shown subsequently, these same treatments yielded significant rank difference correlation coefficients when ranked according to the percentage of hyacinths sunk.

Rate of killing based on the number of days required to obtain the maximum percentage of plants killed was also a function of the concentration of 2,4-D and its rate of application (Table VII). The values for rate of killing are more widely dispersed than those for percentage of plants killed. Rate of killing was frequently more closely associated with rate of application of 2,4-D (lb./acre) than with the concentration. For example, a time period for maximum killing of 41 days or longer occurred at all four concentration ranges, whereas none in excess of 41 days occurred at rates greater than 4 lb. per acre (Table VII). The average number of days for all dates was significantly different for the two highest and two lowest

TABLE VII

RELATION BETWEEN CONCENTRATION AND RATE OF APPLYING THE AMINE SALT OF 2,4-D WITH RESPECT TO THE NUMBER OF DAYS REQUIRED TO OBTAIN MAXIMUM KILLING OF WATER HYACINTH

Date of treatment	According to percentage concentration				According to lb./acre			
	0.16–0.49	0.50–0.99	1.00–1.99	2.00–15.6	2	4	6–8	16–20
May 31 to June 8	21 36 37 37	21 25 31 44 37	25 8 36 13	13	36 37 31 36 37 44	21 25 25 37	21 8 13	13
Av.	33	32	21	13	37	27	14	13
June 24 to July 6	57 57 53 50	53 28 28 41	41 25 21 21 50	39 21 15 50 50	57 53 41 57	39 28 25 53 50 41 50 50 50	21 28 21	15 21
Av.	54	38	32	35	52	43	23	18
Aug. 13 to Aug. 31	39 48	25 39 39 41 41	25 28 28 21	17 35 55 39 35 28	39 48	25 39 28 35 39 55 41	25 28 21 39 41 35 28	17
Av.	44	37	26	35	44	37	31	17
Av. for all treatments	44	35	26	33	43	38	25	17

concentration ranges as well as rates of application, but the differences between either the two lowest or two highest ranges of concentration or rates of application were not significant. These results indicate that a marked increase in the rate of killing is not likely to be accomplished by a slight to moderate increase either in concentration or rate of application. It would appear that a significant increase in rate of killing would require an increase in concentration or rate of application of more than two-fold.

The grouping of values in Table VII according to date of treatment was done for the purpose of representing all four ranges of concentration and rates of application. On the basis of the three date groups the average number of days was relatively the same for the four rates of application and hence yielded a low and non-significant *F* value for the interaction (date of treatment × rate of application). The corresponding interaction

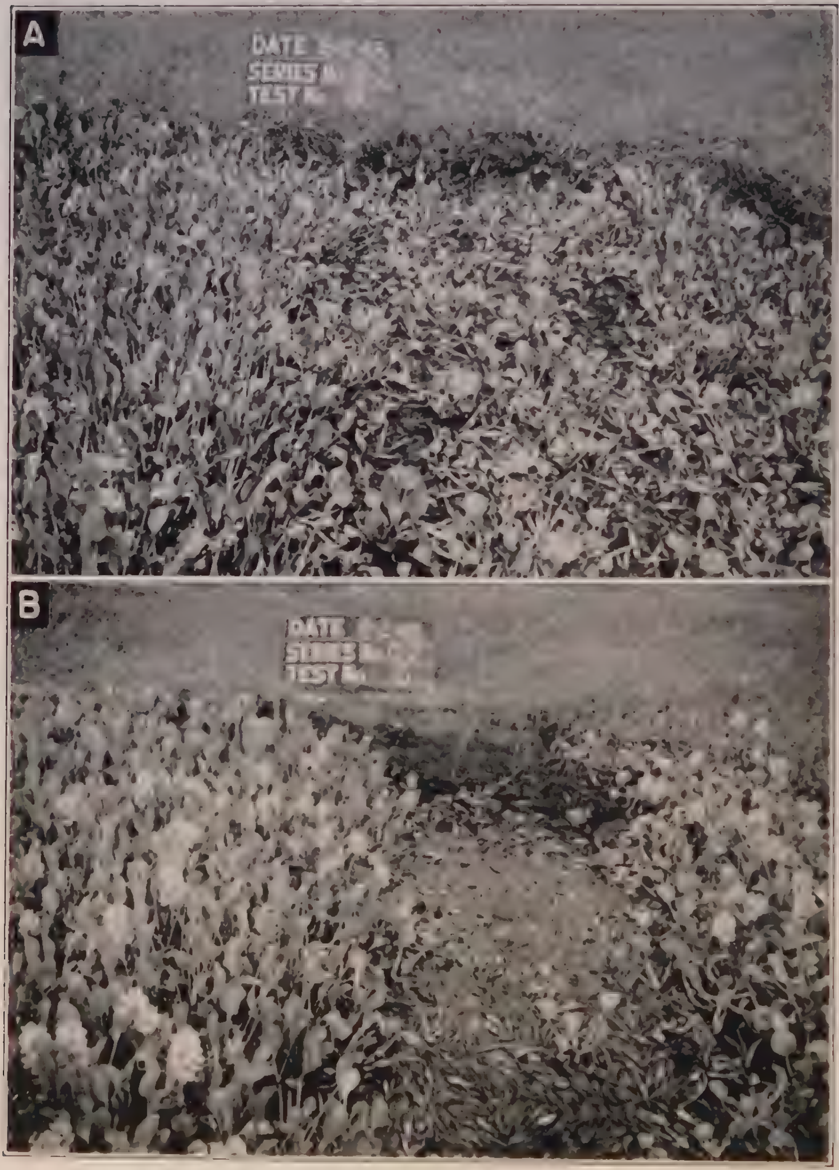

FIGURE 4. Results after 24 days (Aug. 2) of 2,4-D amine salt applied June 8 to hyacinths at ends of pits at rate of 57 gal./acre: A. 0.1 per cent (0.5 lb./acre); B. 0.32 per cent (1.5 lb./acre). The lowest dose was ineffective since only 50 per cent of the plants were killed and subsequently there was a 90 per cent regrowth and reinfestation.

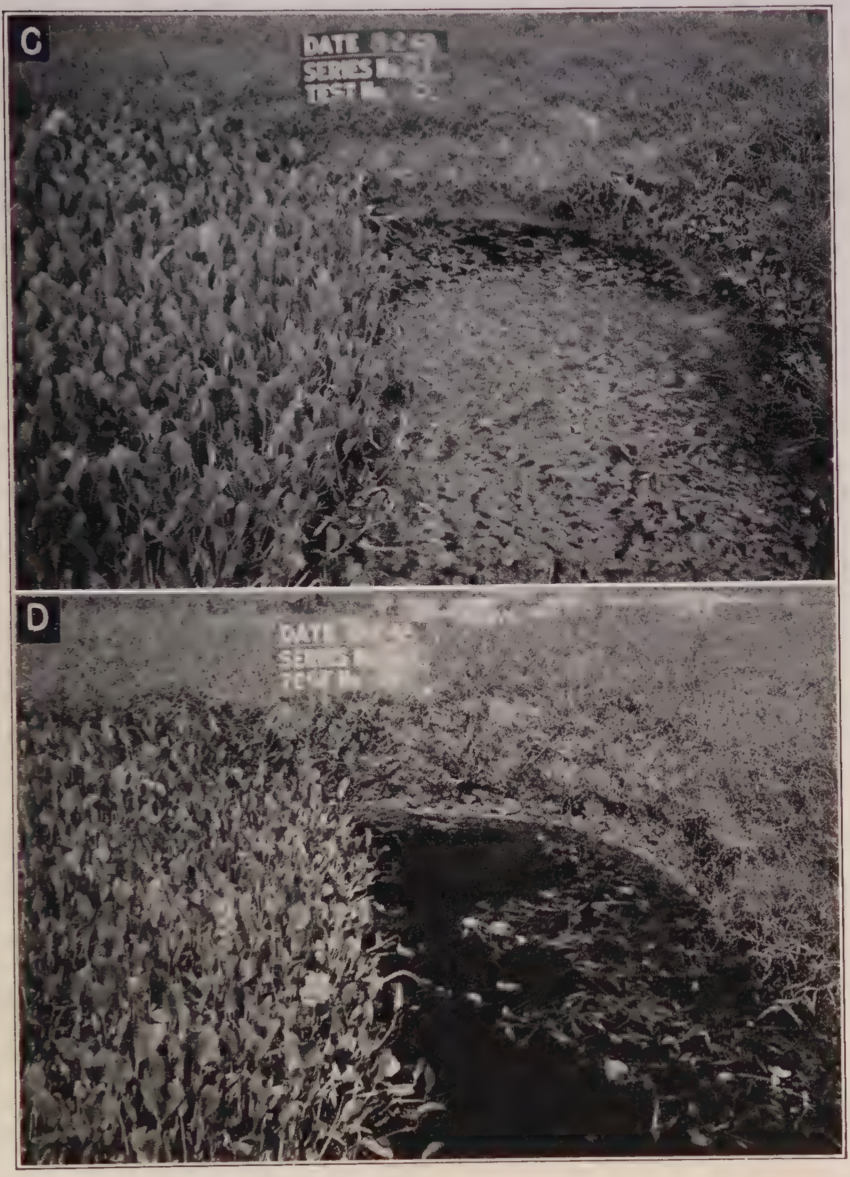

FIGURE 4 (*continued*). C. 1.0 per cent (4.75 lb./acre); and D. 3.2 per cent (15.2 lb./acre).

(date of treatment × concentration) yielded a significant *F* value as might be anticipated from the inconsistent average values obtained on different dates (Table VII). Differences associated with date of treatment were also reflected in the number of plants not killed and the percentage of hyacinths sunk in which case the treatments applied August 17 were more effective than those applied June 24 (Tables IV and V). During the period May 31 to August 31 the rate of killing increased more consistently with increasing rates of application than with increasing concentration of 2,4-D (Table VII). Hyacinths with roots in soil were killed more readily than plants with all roots in water.

When there was adequate coverage, as for example with delivery rates of 43 to 70 gal. per acre, ineffective killing occurred if either or both concentration of 2,4-D and rate of application was relatively low. Results of an ineffective treatment which killed less than 25 per cent of the hyacinths are shown in Figure 4 A, in which case the concentration of 2,4-D was 0.1 per cent and the rate of delivery 0.48 lb. per acre. At the end of 70 days a nearly complete reinfestation had occurred as a result of the growth of plants not killed and their rapid reproduction by means of offshoots.

Sprays of the 2,4-D amine salt applied on October 15 with a Bean gun sprayer operated at 400 lb. pressure from a boat, resulted in adequate coverage of about 80 per cent of an irregular fringe growth of hyacinths covering an estimated 16 acres along the borders of a canal (borrow pit). In this case a 1 per cent solution of 2,4-D delivered at the rate of about 100 gal. per acre caused killing and at least 90 per cent of all plants adequately covered sank by the end of six weeks. Hyacinths in one of the experimental pits were sprayed with a 1 per cent solution of the 2,4-D amine salt by means of the Bean gun operated 5 ft. from the end of the pit and delivering the spray at the rate of approximately 8 lb. per acre. After 44 days all of the plants within 25 ft. of the sprayer were killed, but only 80 per cent of those located 25 to 40 ft. away, with the result that eventually 80 per cent reinfestation occurred.

During January, February, and March, 1949, hyacinths in borrow pits were sprayed with the amine salt of 2,4-D when temperatures were 0° to 27° C. The sprays were applied at the rate of about 100 gal. per acre with a Bean gun operated from land or from a boat. Results of these tests show that 80 per cent or more of the sprayed hyacinths were killed regardless of the temperature at the time of treatment. Differences in the percentage of hyacinths killed and in the rate of killing were more pronounced according to the dose of 2,4-D (2 to 10 lb. acre) than to differences in temperature at the time of treatment for any given dose. It was evident in these as well as in previous tests with the low pressure spraying equipment that an application of 8 lb. or more of 2,4-D per acre caused a more uniform and a faster rate of killing than a 2 lb. per acre rate. In view of these results it is apparent that spray operations for the practical control of hyacinths can be carried on throughout the year. One outstanding advantage of winter-season spraying is that reinfestation of treated areas occurs at a very slow rate, thus providing for a much wider latitude in patrol maintenance operations than is possible when sprays are applied during the summer at a time when hyacinths are in an active stage of growth and vegetative reproduction.

TABLE VIII

RELATIVE EFFECTIVENESS OF ESTERS AND AMINE SALTS OF 2,4-D AND 2,4,5-TRICHLOROPHENOXYACETIC ACIDS

Date treated	Code name of ester and salt	Concn., %	Rate applied, lb./acre	Results after days	Top kill, %	Regrowth, %
May 31	2,4-D isopropyl ester	0.55	2	45	100	10
	Allyl α-(2,4-dichlorophenoxy) propionate	0.55	2	45	100	10
	2,4,5-T isopropyl ester	0.55	2	45	90	90
	2,4-D amine salt	0.55	2	45	100	5
	2,4,5-T amine salt	0.55	2	45	99	75
June 1	2,4-D isopropyl ester	0.55	2	44	99	1
	2,4-D amine salt	0.55	2	44	99	2
June 9	2,4-D isopropyl ester	0.16	2	36	85	25
June 24	2,4-D isopropyl ester	0.64	8	41	100	<1
	2,4-D amine salt	0.64	8	41	100	<1
	2,4,5-T isopropyl ester	0.64	8	41	90	90
	2,4,5-T amine salt	0.64	8	41	99	25
July 20	2,4-D isopropyl ester	1.2	8	41	100	0
	2,4-D butyl ester	1.2	8	41	100	<1
	2,4-D allyl ester	1.2	8	41	100	0
	2,4-D amine salt	1.2	8	41	100	0
	2,4,5-T isopropyl ester	1.2	8	41	100	0
	2,4,5-T amine salt	1.2	8	41	100	1
July 27	2,4-D isopropyl ester	0.64	8	38	100	0
	2,4-D amine salt	0.64	8	38	100	0
Aug. 31	2,4-D amine salt	0.32	2	50	100	1
	2,4,5-T amine salt	0.32	2	50	95	15

Results obtained with the Bean gun sprayer showed that effective killing of hyacinths occurred only within a distance of about 25 ft. Attempts to spray beyond the effective range of the sprayer or to respray lightly covered or missed areas by reducing the speed of the spray rig below three miles per hour or by backing up the boat or tractor, resulted in an ineffective killing even when twice the quantity of spray solution (200 gal./acre) was used. It is considered more efficient in practical control operations to make one spray trip over a given area with an effective dose of 2,4-D than to attempt to cover a wider area by increasing the rate of delivery. Plants not killed by the first application used on a single trip can be sprayed one to two months later as part of a patrol maintenance program whereby a second or follow-up spray should be applied before noticeable reinfestation occurs.

Esters of 2,4-D. The isopropyl and butyl esters of 2,4-D were of about equal effectiveness to the amine salt in killing 90 to 100 per cent of the hyacinths (Tables VIII and IX). Hildebrand (7) also reported equivalent results for the salts and esters of 2,4-D. In one series of tests the allyl ester of 2,4-D caused a slightly faster killing and a higher percentage of sinking than either the isopropyl or butyl esters. The allyl ester of α-(2,4-dichlorophenoxy)propionic acid was less effective than that of the acetic acid homolog. Results obtained with the 8 lb. per acre rate are shown in Table IX.

TABLE IX

RELATIVE EFFECTIVENESS OF THE APPLICATION OF 8 LB./ACRE OF DIFFERENT FORMULATIONS FOR KILLING AND SINKING HYACINTHS AS NOTED AFTER 41 DAYS

Formulations containing 1.2% 2,4- or 2,4,5-chlorophenoxy compounds	No. of plants present	% Plant material sunk
2,4-D isopropyl ester	0	60
2,4-D butyl ester	1	60
2,4-D allyl ester	0	95
2,4-D alkanolamine salt	0	85
2,4-D isopropyl ester + 1% Na-trichloroacetate	0	75
2,4-D isopropyl ester + 3.2% Na-trichloroacetate	0	100
2,4-D isopropyl ester + 10.0% Na-trichloroacetate	0	100
2,4,5-T isopropyl ester	0	50
2,4,5-T alkanolamine salt	2	50

Esters and salts of 2,4,5-T. At rates of less than 8 lb. per acre the amine salt and isopropyl ester of 2,4,5-T were considerably less effective for killing hyacinths than the corresponding 2,4-D analogs (Table VIII). In the 8 to 16 lb. per acre range the two trichlorophenoxy formulations were of about equal effectiveness to the amine salt of 2,4-D for killing hyacinths, but less effective for sinking the plants (Tables VIII and IX). Even at the 8 lb. per acre rate a concentration of 0.64 per cent was much less effective than 1.2 per cent (Table VIII).

Non-hormone herbicides. Several non-hormone toxicants were used alone and in combination with 2,4-D. All of these toxicants (Table X) were less effective or ineffective for killing hyacinths when used at the approximate minimum effective dose of 2,4-D. None of the toxicants, except possibly sodium trichloroacetate, caused noticeable additive effects when used in sub-lethal quantities in combination with 2,4-D. In fact, these mixtures were generally less effective than the equivalent dose of 2,4-D, indicating that the non-hormone toxicant may have interfered with the penetration or transport of 2,4-D. Non-hormone toxicants which killed or severely injured the foliage of hyacinths failed to prevent the development and opening of flower buds, as did effective doses of 2,4-D.

TABLE X

RELATIVE TOXICITY OF NON-HORMONE HERBICIDES ON HYACINTHS

Herbicide	Concn., %	Gal./acre	Initial top kill	Plants killed, %
Benoclor 3C	10.0	43	Trace	0
Pentachlorophenol	0.32	43	5%	0
	0.1	70	Trace	0
Dow General Weedkiller (dinitro)	1.0	70	95%	0
Hammond's Weedkiller (arsenical)	5.0	70	100%	>99
Sodium trichloroacetate (Dow)	10.0	57	50%	0
	3.2	57	Trace	0
Sodium chlorate	12.0	800	100%	100

Results indicating possible additive effects with sodium trichloroacetate are shown in Table IX. Similar results were obtained with a mixture of 0.84 per cent 2,4-D amine salt and 1.0 per cent sodium trichloroacetate applied on August 18 to an entire pit of hyacinths. In this case all but one of the approximately 2,000 plants were killed and 95 per cent were sunk at the end of 63 days. Ends of pits containing hyacinths were also treated with similar mixtures on the same day. For example, a mixture containing 0.84 per cent 2,4-D and 3.2 per cent trichloroacetate killed all but one of about 350 plants but caused sinking of only 25 per cent. A concentration of 0.84 per cent 2,4-D applied at the rate of 4 lb. per acre killed all hyacinths but caused sinking of only 10 per cent in 63 days. A 10 per cent solution of sodium trichloroacetate, which was not completely soluble, caused neither killing nor sinking. Successive treatments after a 12-day interval with either 0.84 per cent 2,4-D or 3.2 to 10 per cent sodium trichloroacetate applied first, resulted in the killing of all plants, but none had sunk at the end of 63 days.

Mixtures of amine salts or isopropyl esters of 2,4-D and 2,4,5-T were of equal effectiveness to 2,4-D used at the total concentration of the mixture, and were more effective than the equivalent salt or ester of 2,4,5-T used alone. These results applied to total concentrations of the mixtures ranging from 0.5 to 1.0 per cent with corresponding rates of application of 3 to

6 lb. per acre. As mentioned previously, the 2,4,5-T formulations did not generally kill as high as 90 per cent of the hyacinths when used at rates of less than 8 lb. per acre and less than 1.2 per cent. It thus appears that the effectiveness of a 2,4-D solution containing 0.32 per cent 2,4-D can be increased to about the same extent by adding additional quantities of either 2,4-D or 2,4,5-T salt or ester up to a point where the total concentration of the mixture is 1.0 per cent.

TABLE XI

RATE AT WHICH HYACINTHS SANK AFTER TREATMENT WITH 2,4-D AMINE SALT APPLIED AT DIFFERENT RATES (LB./ACRE) TO ALL PLANTS IN A PIT ON AUGUST 13, 1948, USING A DELIVERY RATE OF 57 GAL./ACRE

Concn., %	Lb./acre	% Plant material sunk after weeks indicated			
		4	6	7	8
3.36	16	70	95	95	100
1.68	8	50	90	95	99
0.84	4	10	70	90	98
0.42	2	0	40	90	98

SINKING OF TREATED HYACINTHS

Mats of dead hyacinths cause mechanical obstructions much the same as live plants. These dead mats of plants also serve as a support for the rapidly growing alligator weed. One of the important phases of practical control is to bring about a rapid disintegration or sinking of the hyacinths treated with 2,4-D. The use of proper doses of 2,4-D caused relatively rapid sinking of the treated hyacinths regardless of whether the plants were entirely dead.

A higher dose of 2,4-D and a longer time period after treatment were required to cause sinking of hyacinths than killing the plants. There was an indication that hyacinths treated in June sank less readily than those treated during July or later in the season. This applied particularly to rates of less than 8 lb. per acre.

Values representing the estimated amount of hyacinths sunk were more widely dispersed than values for the percentage of plants killed. The same treatments previously mentioned which yielded non-significant correlation coefficients (r') for the relationship between percentage of hyacinths killed and either concentration or rate of application, yielded significant r' values when the percentage of hyacinths sunk was used instead of the percentage killed. Thus the r' values for concentration were .9231 and .8842 respectively by Spearman's and Friedman's methods. The corresponding r' values for lb. per acre were .7411 and .6292. In contrast, non-significant negative r' values were obtained for rate of delivery (−.2802 and −.4602). The magnitude of the negative r' values indicates that low gallonage sprays may have been slightly more effective than the high gallonage sprays, mainly on the basis that low volume delivery was generally associated with the use of relatively high concentrations and high rates of applications (Tables I, VI, and VIII).

The influence of concentration and rate of application were more pronounced on the rate of sinking than on the final percentage of hyacinths sunk (Tables V and XI). However, at a given rate of delivery, the final percentage of plants sunk generally increased with increasing concentration or rate of application (Tables IV and V and Fig. 4). For example, at the highest delivery rate of 70 gal. per acre the percentages of hyacinths sunk at the end of 67 days were 25, 50, 85, and 95 respectively for spray concentrations of 0.16, 0.32, 0.64, and 1.28 per cent (Table IV). A similar relationship held also for the other rates of delivery. With a fixed rate of delivery the influence of an increasing rate of application was, of course, the same as for corresponding increases in concentration (Fig. 4).

When the rate of application of 2,4-D was held constant either at 4 or 8 lb. per acre, and the rate of delivery was increased with a proportional decrease in concentration, there was no marked difference in the percentage of hyacinths sunk, any differences being in favor of the intermediate rates of delivery and not the highest rate of 70 gal. per acre (Tables IV and V). These results indicate that any appreciable increase in the percentage of hyacinths sunk is not likely to be accomplished by increasing the rate of delivery considerably above 57 gal. per acre for a fixed rate of application in the range 2 to 4 lb. per acre, since such a procedure would necessitate a proportional decrease in concentration of 2,4-D to the range below 1.0 per cent which proved ineffective for sinking (Table IV and Fig. 4). On the other hand, if the concentration of 2,4-D is fixed at 1 per cent, increasing the rate of delivery from 6 to 150 gal. per acre would be expected to cause an increased percentage of sinking due to an increase in rate of application from 0.5 lb. to 12.5 lb. per acre. The results in this case would be similar to those shown in Figure 4 which illustrates the effect of increasing the rate of application from 0.5 to 15.2 lb. per acre simultaneously with an increase in concentration from 0.1 to 3.2 per cent while the rate of delivery was held constant at 57 gal. per acre.

The complete killing and sinking of all treated hyacinths resulted from certain treatments applied to entire pits on August 13 (Table XI and Fig. 5 A) and to ends of pits on August 17 (Table V and Fig. 5 B). Although rates of 2 to 4 lb. per acre generally did not cause sinking of all treated plants, these lower rates appeared to be more effective when administered after maximum growth and the rate of reproduction by offshoots had been attained (about August 1). The factors mainly responsible for causing hyacinths to sink are not known at present. Maceration of most treated plants usually occurred, but complete maceration of the entire plant was not essential for sinking, since entire plants and parts of plants

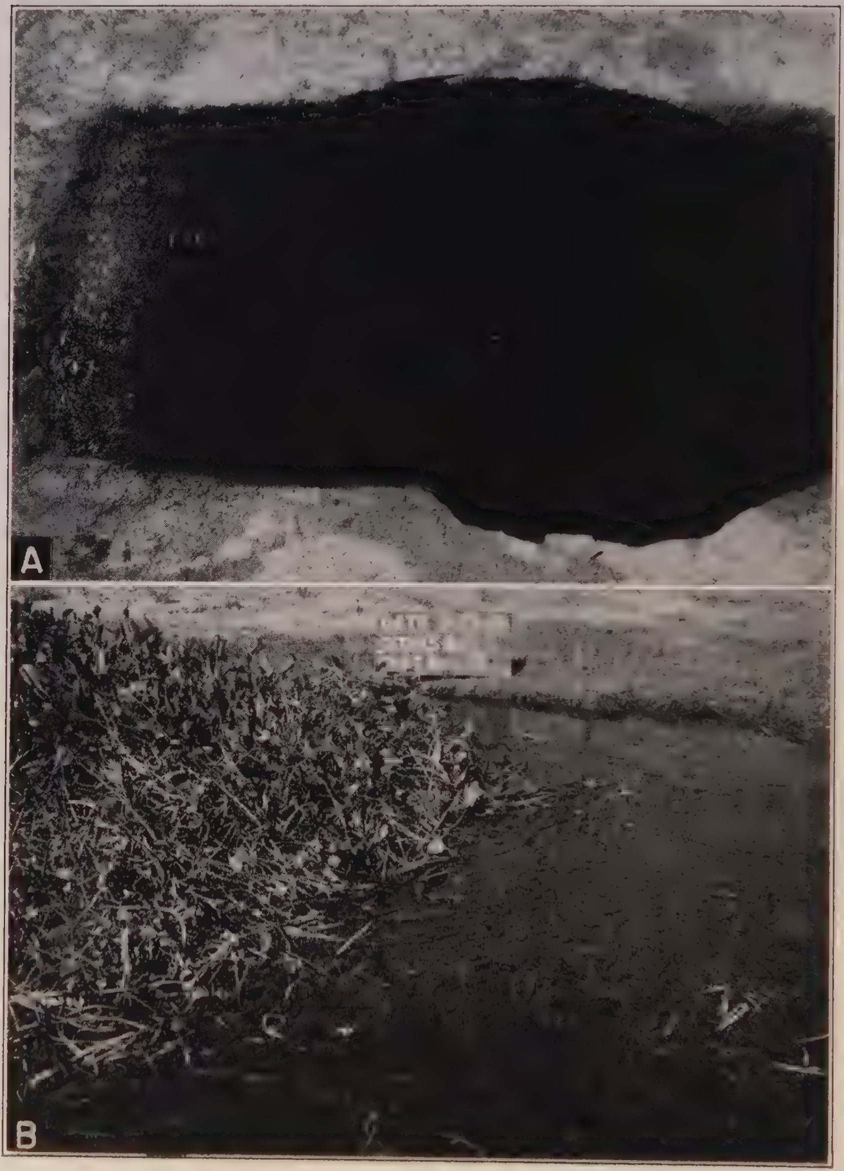

FIGURE 5. Relative effectiveness on September 29 of the same 2,4-D treatment applied (A) to an entire pit on August 13, and (B) to the end of a pit on August 17; 1.68 per cent 2,4-D amine salt was applied at the rate of 57 gal./acre (8 lb./acre). All treated hyacinths were killed and the percentage of plants which sank was 99 in A and 100 per cent in B.

sank and remained unmacerated for several days or longer. In some cases a partially macerated crown with most leaves attached, like the plant on the right in Figure 3 A, sank after becoming separated from its root system. Similar sinking of partially macerated stems of alligator weed was also observed, but with the difference that the main masses of roots were still attached in most cases to stems that were one to several feet long and consequently large masses of plant material several to many feet in diameter sank as a unit.

The rate of maceration and also of sinking was presumably associated with the presence of 2,4-D. When non-treated hyacinths died, as in the case of those which were shaded with canvas, maceration was much slower and the dead plants did not sink during a three-month period after 90 per cent of the aerial parts were dead (September 20 to December 16). However, all of the dead plants sank eventually and at a slow rate after the middle of December. Once the sinking of hyacinths started, the process continued at a rapid rate in the case of effective treatments applied during the summer. For example, all treatments causing from 50 to 95 per cent sinking at the end of 41 days (Table IX) had caused this amount of sinking within eight days (34th to 41st day).

Hyacinths killed by 2,4-D sprays applied in January (Fig. 6) and February appeared to macerate more slowly and required a longer time to sink than those killed during the summer, but the comparative data have not yet been fully evaluated. Effective sprays applied during winter appeared to cause a higher percentage of entire plants to sink than was true for summer sprays. The rate of sinking of winter-treated plants was faster for the highest doses of 2,4-D than for the lowest doses as in the case of summer sprays.

REINFESTATION OF HYACINTHS IN CONTROL AND TREATED AREAS

Some knowledge of the normal rate of growth and vegetative reproduction of hyacinths at different times of the year is necessary in order to comprehend the pronounced differences in reinfestation following effective and ineffective treatment with 2,4-D. For example, three hyacinths placed in a pit on May 11 produced approximately 3,000 new plants in 50 days and about 7,500 at the end of three months (August 15). This is to be contrasted with the production of 32 new plants in 50 days from three hyacinths placed in a pit on August 23. About August 15 the older extensively developed roots up to two and one-half feet long began to die on plants growing under natural conditions. By October 1 the root systems of most plants were greatly reduced in size and poor in appearance. It was estimated that the rate of growth of hyacinths and their vegetative reproduction were reduced to at least 20 per cent after August 15. Consequently, many of the treatments applied during June and July which resulted in

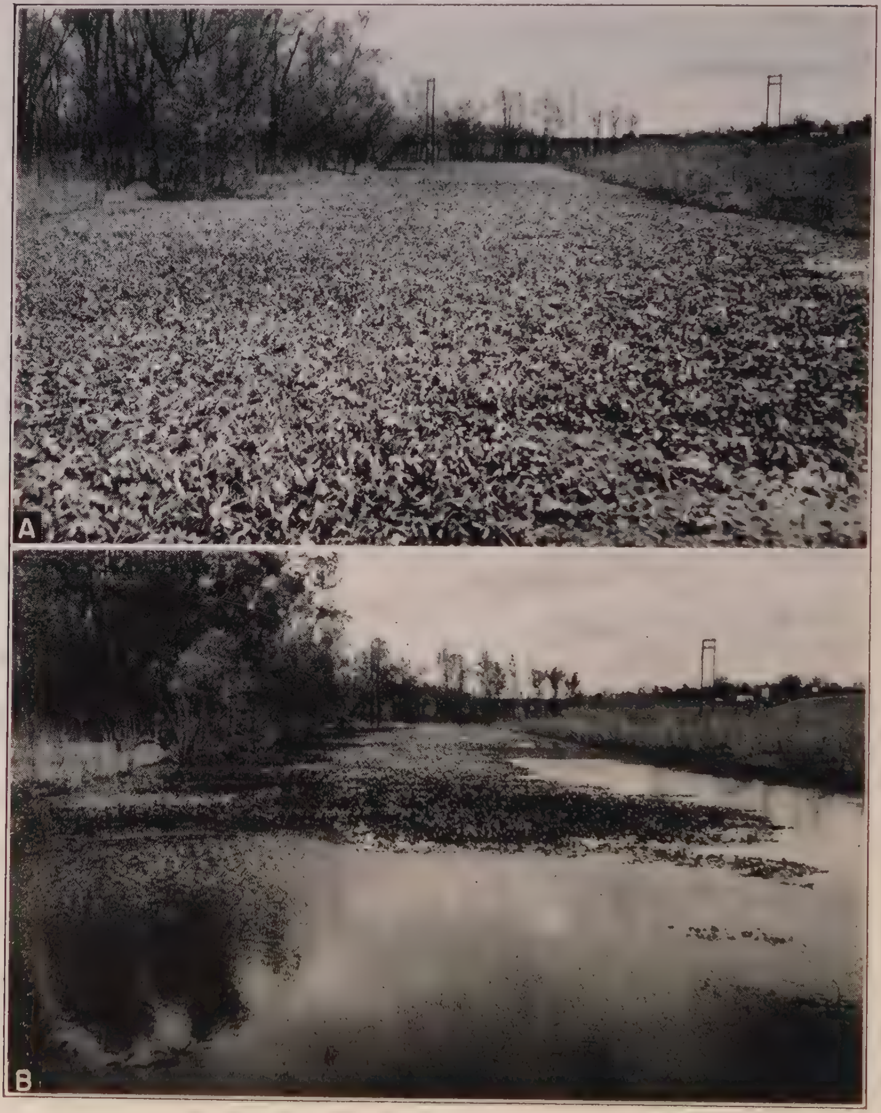

FIGURE 6. Killing and sinking of tightly packed hyacinths 12 to 24 in. tall resulting from treatment on January 7, 1949 (air temperature of 18° C.) with 1 per cent 2,4-D alkanolamine salt delivered at the rate of 100 gal./acre (10 lb./acre) by means of a Bean gun operated at 300 to 400 lb. pressure from both banks. A. Appearance of dead hyacinths February 24, 1949, when sinking started. B. Appearance on March 28, 1949, when about 50 per cent of the treated plants had sunk.

less than 100 per cent killing appeared less effective than they actually were due to the relatively rapid rate of growth and production of offshoots on plants which were not killed.

Ineffective treatments causing less than 90 per cent killing permitted various amounts of regrowth or reinfestation up to a complete filling in of the treated area in the course of two to three months (Fig. 4 A). This type of regrowth consisted of a resumption of growth and the production of new leaves and new plants from surviving parts of plants (old rhizomes) even though most or all of the above-water parts may have been killed or badly damaged. Many of the newly developed leaves were modified. In the case of effective treatments causing 90 to 99 per cent killing, the surviving plants were very small and grew at a slow rate. These small plants were not seedlings as evidenced by the piece of attached stolon and the presence of leaves having normal characteristics of adult plants. No seedlings were found in any of the 108 pits during the period May to October inclusive, but a considerable number were found in April, 1949. Presumably these "plantlets" formed at or slightly above water level had not received an effective dose of 2,4-D as a spray or by transport from the parent plant through the stolon. The surviving plantlets did not exhibit any typical hormone responses other than a retarded growth.

As mentioned previously, in special tests with individually treated hyacinths there was little or no transport of 2,4-D from parent to daughter plants, or vice versa, in the case of low concentrations (less than 0.3 per cent), but there was some transport with a concentration of 1 per cent, although in these particular tests not enough to cause killing. It has not been fully established whether the failure of plantlets to survive in areas where 100 per cent killing was obtained is due to direct killing by the spray or from transport of 2,4-D through stolons.

Practical applications. Since complete killing and sinking of all hyacinths resulted from certain treatments with 2,4-D under conditions where good coverage was obtained, it seems likely that with the development of special spray equipment and the use of proper methods of application the large scale practical control of hyacinths in Louisiana may be accomplished. On the basis of the results obtained to date, it appears that practical control of water hyacinth, in areas which are accessible to boat-mounted cutting machines (saw-boats), might be accomplished by a system of patrol maintenance whereby the remaining fringe of plants not chopped up by saw-boats could be killed by an effective dose of 2,4-D applied before any large scale reinfestation had occurred. This would no doubt necessitate the use of two or more sprays each year. Considering the marked seasonal differences in the rate of growth and reproduction of hyacinths, the timing of spray applications would be of considerable importance.

In view of the favorable results obtained with winter sprays, it is believed that practical control of hyacinths can be most effectively accomplished by applying the initial spray when the plants are not in an active stage of growth. This would mean applying the initial spray any time from about the middle of August to about the first of March. Spraying of hyacinths during their active growth period is of course not precluded, but the relatively rapid rate of growth and reproduction of plants which are not killed, makes patrol maintenance spraying during spring and summer more critical with respect to the timing of the subsequent sprays. Hyacinths which reinfested areas treated with 2,4-D after August first were relatively small in size until the following spring. Small plants less than one foot tall are covered adequately and more readily with a smaller quantity of spray solution, and they are also more readily killed, than the large plants which exceed one foot in height.

Practical control of hyacinths with 2,4-D will depend upon the efficient killing of at least 80 per cent of the plants in treated areas. By efficient killing is meant application of an effective dose of 2,4-D within the effective range of the sprayer so that the plants are adequately covered in a single trip with a spray solution delivered at the rate of about 100 gal. per acre. Any attempt to spray plants beyond the predetermined effective range of the sprayer, or to respray inadequately covered areas by reducing the speed of operation much below three miles per hour or by backing up the spray rig, would constitute inefficient treatment involving a waste of materials and time which would increase the cost of the initial and subsequent spray operations.

Areas occupied by hyacinths generally have irregular margins. Even in the case of efficient saw-boat operations an uncut fringe of plants is usually left along the margins of the waterway in question. Prompt treatment of these fringes of hyacinths with an effective dose of 2,4-D must be made in order to prevent reinfestation of the area cleared by saw-boats. Although the width of the fringe may vary from less than one foot to more than 25 feet, it would not generally be considered desirable to make more than one spray trip for the initial application of 2,4-D. The hyacinths not reached or adequately covered by the first spray can be killed by subsequent patrol maintenance sprays. It has not yet been determined whether sprayers can be operated as effectively from saw-boats as from boats equipped only for spraying.

Regardless of the extent of the area of hyacinths to be treated, the method of spraying is essentially the same, namely, to reduce the area systematically by spraying successively in strips equal in width to the effective range of the sprayer. Since hyacinths receiving an effective dose of 2,4-D exhibit pronounced hormone responses within five days, it is possible after this interval of time to distinguish between plants which have received an adequate dose of 2,4-D and those which have not. Consequently, a second spray can be applied any time after the five-day period up to the time when noticeable reinfestation of cleared areas starts.

A new type of nozzle manufactured by the Spraying Systems Company appears to offer much better possibilities for use in killing hyacinths than any other nozzle with which the authors are acquainted. This nozzle is of the Teejet type having a 1-¼ inch connection and is designed to deliver a flat off-center pattern of spray for distances up to 21 ft., depending upon the size of the opening, the pressure, the distance it is held above ground, and the pump capacity in gallons per minute (G.P.M.). Preliminary coverage tests with the nozzle designed to deliver about 7 gal. per minute at 60 lb. pressure indicate that distances up to nearly 40 feet can be reached when the pump discharges 15 or more G.P.M. Various types of power equipment are now being tested in order to determine the maximum distance which these nozzles will discharge a flat spray at delivery rates of about 100 gal. per acre and a rig speed of about four miles per hour. If the effective range of this nozzle proves to be 25 feet or more, it will represent a distinctly new stage of development in the spraying of hyacinths as well as other species of plants growing in water or on land.

RESULTS WITH ALLIGATOR WEED

The most effective sprays applied to the ends of pits containing alligator weed caused considerable damage but failed to kill the plants. Since some of these same treatments were effective in killing and sinking hyacinths, it was apparent that the alligator weed was going to be much more difficult to kill than the hyacinth. A possible clue to an effective treatment on alligator weed was obtained from the results of tests in which all of the plants in a pit were sprayed on August 31 in one case with a 17.5 per cent solution of the isopropyl ester of 2,4,5-T applied at the rate of 8 lb. per acre with the 6 gal. per acre nozzle, and in another case with the amine salt. Within one month most plants treated with the ester were badly damaged and many had sunk below the surface of the water. In three months all plants had sunk without exhibiting any signs of regrowth. At the end of six months some regrowth was evident at the shallow end of the pit. It is not certain whether the effectiveness of this treatment is due to the time of year or to the fact that all plants were sprayed, including those anchored to the banks of the pit. Results with hyacinths treated after July also indicated that spraying entire pits was more effective than treating the ends. Sprays delivered by the lowest volume nozzle (6 gal./acre) appeared to be more effective on alligator weed than those delivered by higher rate nozzles. The reverse was true for hyacinth.

In contrast to the ester, the amine salt of 2,4,5-T caused killing of all leaves but relatively little sinking of the stems of alligator weed. After six months no regrowth was found in the pit sprayed with the amine salt. The relative effectiveness of the salt and ester cannot be finally evaluated until it has been determined whether the salt caused temporary or permanent inhibition of bud growth.

GREENHOUSE AND LABORATORY EXPERIMENTS

Growth requirements of hyacinths in the greenhouse. During the period November to March, hyacinths made a satisfactory growth, produced offshoots, and flowered in unshaded greenhouses when the temperature was 21° to 27° C. (70° to 80° F.) and the water contained ample nutrients. Water temperatures during the middle of the day varied from 20° to 34° C. In distilled water, hyacinths failed to grow, became progressively more chlorotic, and finally died. The plants lasted much longer in tap water, but they grew slowly and produced few or no offshoots or flowers. Oxygen was not a limiting factor since aerating the water with oxygen did not improve growth. Satisfactory growth of hyacinths occurred in tap water to which a composted soil was added at the rate of about 150 cc. (100 to 150 g. air-dry basis) of soil per liter of tap water. Soil from the bottom of a pond was not satisfactory. As Bailey pointed out (1, p. 1105) "a good soil underneath" is necessary for satisfactory growth of water hyacinth in small pools or in containers. The difference in growth of hyacinths in tap water with and without soil is shown in Figure 7 B.

The type of container appeared not to be an important limiting factor for growth of hyacinths so long as the water level was maintained within about one inch of the rim of small vessels. In the case of 1- to 2-liter containers water was added daily, and about once a week to containers of 15- to 50-liter capacity. The composition of the container was not an important limiting factor with respect to glass, enamel, or glazed crockery. Depth of water ranged from 4 to 24 inches in the various containers.

Hyacinths which had remained in tap water for several weeks responded in one week to the addition of small quantities of Hoagland and Arnon's complete nutrient plus minor elements (9). Chlorotic or light green leaves became darker green and growth of the young leaves was accelerated. After two weeks most leaves were of nearly normal green color, newly developed leaves were larger, and the production of offshoots was about the same as for plants in water plus soil. Similar plants placed in water plus soil also responded to the addition of a complete nutrient. In this case growth started sooner and the leaves were darker green than those on plants in tap water plus nutrient. By the end of the second week the leaf blades and upper sides of inflated petioles were dark green in color, but growth was not retarded.

Growth response of hyacinth to added nutrient increased with increasing quantities of nutrient, regardless of whether soil was present in the containers. The stock nutrient normally used in water culture experiments was

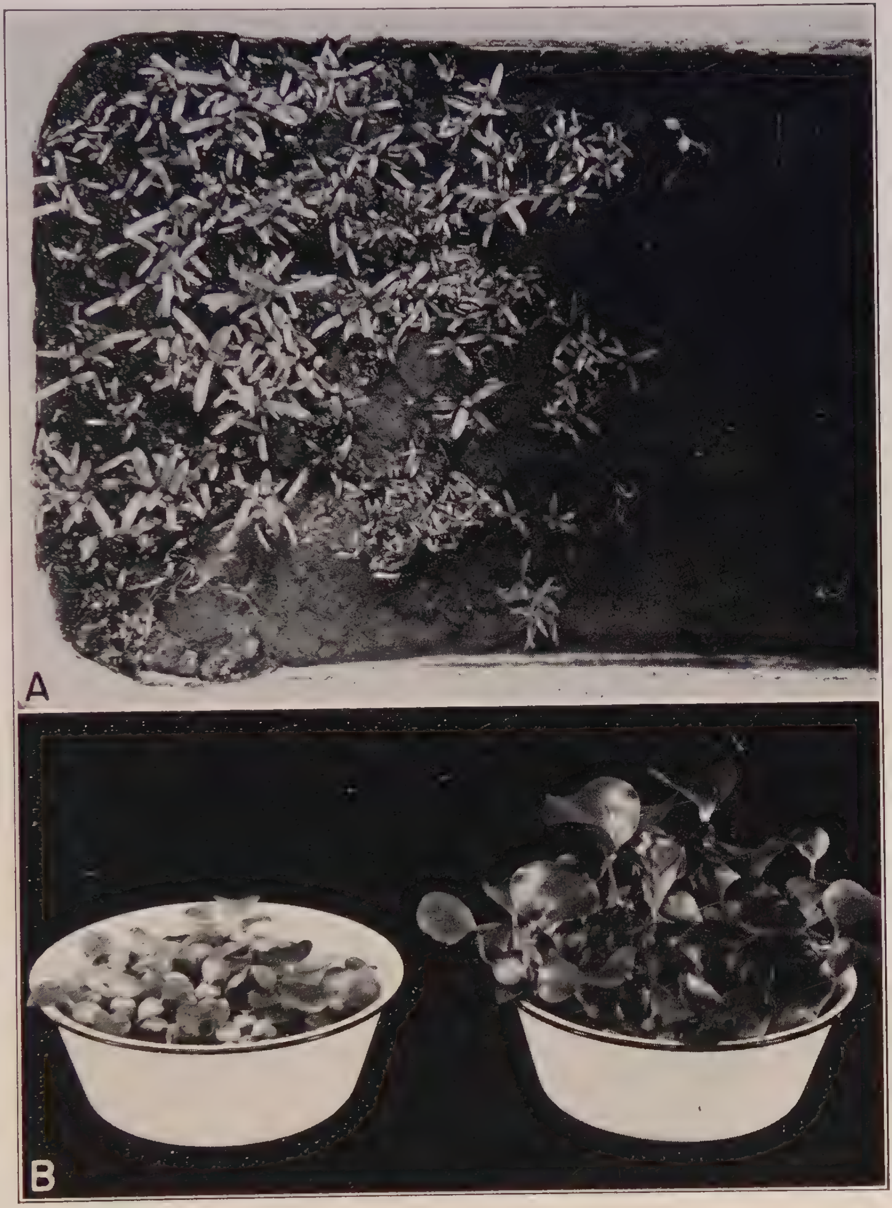

Figure 7. A. About 700 hyacinth seedlings growing in soil which slopes into shallow water at right. B. Growth of adult hyacinths after two months in tap water (left) and in tap water plus soil (right).

added to different containers at the rate of 1, 3.2, and 10 cc. per liter of tap water on the first, third, and seventh days and thereafter weekly. The total concentration of nutrient was from 0.01 to 0.001 as strong as is normally used in water culture experiments. Although optimal nutrient requirements have not been determined, it is evident that the hyacinth has relatively low nutrient requirements as compared with many land plants.

The color of hyacinth roots was an approximate measure of the relative concentration of nutrients in the solution. Roots in tap water were blue or purple and in the case of young seedlings the lower surfaces of leaves in contact with the solution were also blue or purple. With increasing quantities of soil or of Hoagland and Arnon's complete nutrient the roots were lighter in color, being white or nearly white in the higher concentrations of nutrient. The blue pigment deposited on the walls of the container was of the indicator type, turning violet with the addition of acid and green with the addition of alkali. The possible influence of pH on the pigmentation of hyacinth roots has not been worked out.

Extra light from a 1,000-watt nitrogen bulb placed close enough to the foliage to increase the air temperature from 21° to 24° C. influenced growth and flowering to some extent (Table XII). Earlier flowering of hyacinths receiving extra light may have been due indirectly to a more advanced vegetative maturity of these particular plants. Control plants and those under the radiant heater flowered in February shortly after the experiment was terminated. Data in Table XII indicate that temperatures of 24° to 27° C. are more favorable to growth than a temperature of 21° C. The influence of temperature was much more pronounced on flowering than on vegetative growth. Hyacinths receiving a night temperature of 16° to 18° C. and a day temperature of 21° to 23° C. did not flower during the period

TABLE XII

GROWTH AND FLOWERING OF FOUR HYACINTHS UNDER THREE DIFFERENT CONDITIONS IN SAME GREENHOUSE

Condition	Av. no. leaves per plant			Av. length of leaves per plant, mm.		Av. width of leaves per plant after 2 wk., mm.	Temp., °C.*		Water pH	No. flowers in 2 mo.
	Original no.	After 1 wk.	After 2 wk.	After 1 wk.	After 2 wk.		Air	Water		
Heater**	6.0	7.2	8.2	79	103	57	27	27	6.25	0
Light†	6.0	7.8	8.5	96	137	74	24	25	6.15	8
Control	6.0	5.8	6.5	63	87	49	21	23	6.6	0

* Greenhouse temperature maintained at 20° to 22° C.

** Radiant electric heater on daily from 8:30 a.m. to 10:30 p.m. during December and 8:30 a.m. to 5:30 p.m. during January.

† 1,000-Watt nitrogen bulb on for same periods as heater.

October to the middle of March, although vegetative growth and the production of offshoots were about the same as when the temperature did not fall below 21° C. day or night.

Residual effects of 2,4-D in water. When individual plants were removed from the experimental pits in Louisiana to be sprayed with 2,4-D by means of an atomizer or a Hudson Junior sprayer, they were killed in a shorter time and with lower concentrations than plants which were sprayed in an undisturbed condition in the pits by means of the four-nozzle boom. Similarly, plants sprayed individually in the greenhouse at Yonkers, New York, during the winter season were killed more readily than masses of plants sprayed in the field in Louisiana during the summer season. Although a more complete coverage may have been attained in the case of individually sprayed plants, it seemed likely that the quantity of 2,4-D spray falling on the surface of the water might produce an added effect in the case of plants in small containers of one- to two-liter capacity.

TABLE XIII

RESIDUAL EFFECT IN ONE LITER OF WATER OF 2,4-D AMINE SALT SPRAYS APPLIED TO HYACINTH FOLIAGE, AND THE COMBINED EFFECT OF THE FOLIAGE SPRAY AND RESIDUE. DEGREE OF INJURY OR ACCELERATION SHOWN BY PLUS SIGNS

Concn., %		Plant in 2,4-D solutions (see column 1)	2,4-D sprays applied on			
				Foliage of plants		
					Plant removed	
2,4-D solutions	2,4-D sprays		Surface of water (10 cc. of spray)	Plant left in solution	To H_2O	Replaced with new plant
0.1	10	Dead	Dead	Dead	Dead	Dead
0.001	0.1	++ Injured*	++ Injured	Dead	Dead	++ Injured
0.0001	0.01	Retarded	Retarded	Dead	++ Injured	+ Injured
0.00001	0.001	++ Accelerated**	+++ Accelerated	Retarded	++++ Accelerated	++++ Accelerated

* Browning of foliage.

** Elongation of leaves associated with proliferation below blade or smaller diameter than normal.

An experiment was set up to determine the effect of residual 2,4-D spray in water. The results are shown in Table XIII. The 2,4-D falling on the surface of the water directly or as a run-off from the foliage produced an additional effect on the sprayed plant which was not removed, caused noticeable injury to a non-treated plant placed in the contaminated water, and delayed the germination of alfalfa seed and retarded the growth of the seedlings during the first 48-hour period after treatment. For example, a 0.01 per cent 2,4-D spray killed the hyacinths which remained in the contaminated solution but did not kill a similar plant which was removed to control water immediately after treatment. The water, which was contaminated by spraying a plant with 0.1 per cent 2,4-D, caused the same amount of injury (++) as a prepared 0.001 per cent solution of 2,4-D. Hyacinths were affected, although not badly injured, by very low concentration of 2,4-D in the water (1 p.p.m. or less). This suggests the use of hyacinth as a sensitive test plant for detecting the presence of small quantities of 2,4-D in water. An alfalfa (*Medicago sativa* L.) seed germination and seedling test proved even more sensitive since germination of seeds in 0.001 to 0.01 p.p.m. 2,4-D amine salt was delayed and root growth of the seedlings noticeably retarded by 0.001 p.p.m. In this case the average length of control roots on alfalfa seedlings after 48 hours was 9.0 mm. as compared with 2.8 mm. for seedlings in 0.001 p.p.m. of 2,4-D. After 24 hours in 2,4-D solutions the percentage germination of alfalfa seed was 21 in 0.1 p.p.m., 65 in 0.001 to 0.01 p.p.m., and 86 in tap water. In the same series of tests seeds of radish (*Raphanus sativus* L.), cucumber (*Cucumis sativus* L.), and Korean lespedeza (*Lespedeza capitata* Michx.) did not germinate as quickly or give as consistent results as alfalfa seed.

Relatively low doses of 2,4-D applied to the foliage of hyacinths or to the water in which they were growing caused elongation of the leaves considerably beyond the normal rate on similar control plants. This effect was obtained in the field and in the greenhouse (Table XIII). Since dry weight determinations were not made, it is not certain whether this type of stimulation is at the expense of dry matter or whether it involves an increase in dry matter. This apparent stimulation of growth was caused by sprays containing 0.1 per cent or less of the 2,4-D amine salt or when the water contained less than 1 p.p.m. of 2,4-D.

Germination of hyacinth seeds. Two collections of hyacinth seeds were made directly from plants growing in the vicinity of Norco, Louisiana. The first collection was made October 26, 1948, and shipped as air-dry seeds or capsules to Yonkers, New York. The second collection of seed made November 22 consisted of seed stalks mostly with intact capsules which were packed in wet moss. This collection of wet seed was placed under three inches of water upon arrival in Yonkers and kept at room temperature in diffuse light until used in special storage or germination tests. There has been no germination of seed in the large stock bottle to date, a period of five months.

During the period December 15, 1948, to February 5, 1949, 22 lots of seed were placed under various germination conditions, including special storage conditions prior to the germination tests. Germination tests were carried out in unshaded greenhouses. In most of the tests, seeds were

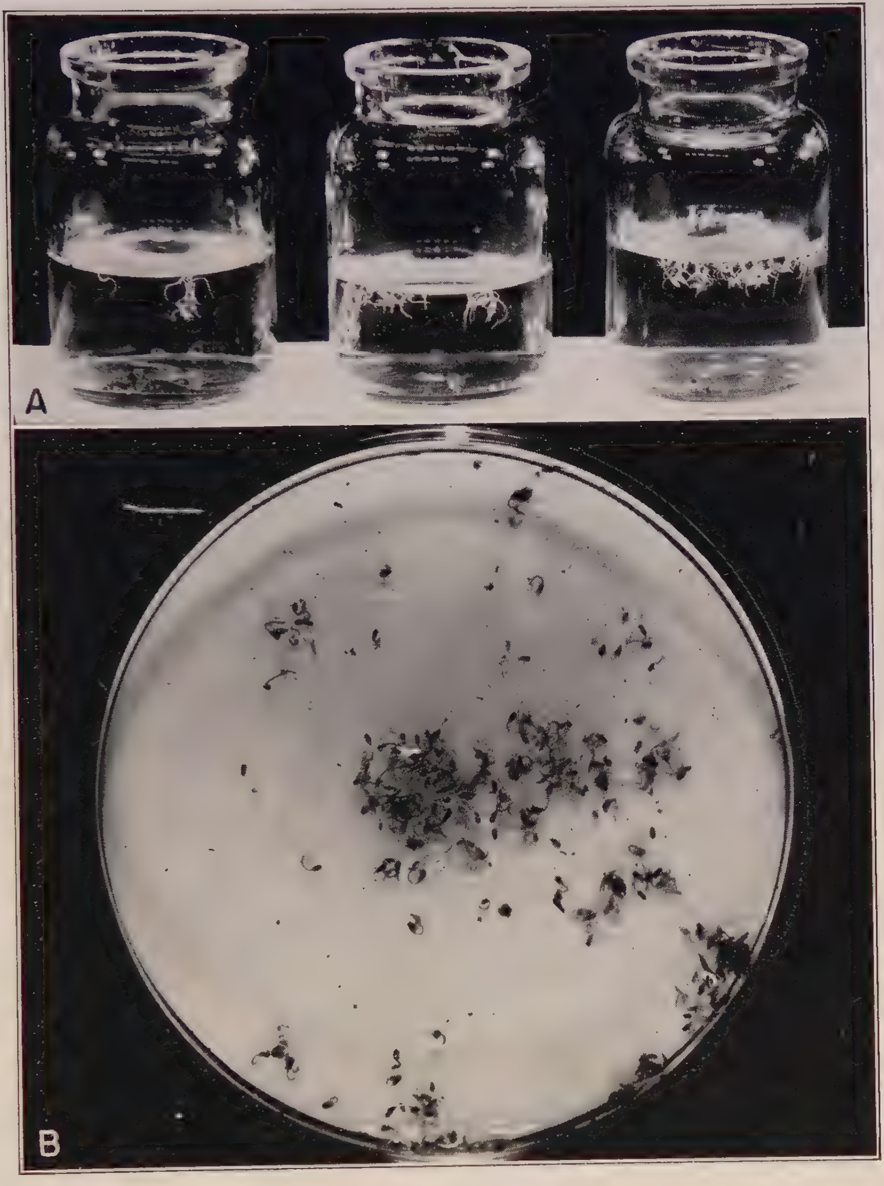

FIGURE 8. Two methods used for germinating hyacinth seeds under water and exposed to sunlight in a greenhouse. A. Seeds germinate on the bottom of the bottle, then rise to the surface after roots and one or more leaves are produced. B. Seeds germinate in shallow water on filter paper in Petri dish, then the paper with seedlings is transferred to saturated soil where paper disintegrates.

placed under tap water which varied in depth from a few millimeters in Petri dishes (Fig. 8 B) to two inches in evaporating dishes or in small wide-mouthed glass bottles (Fig. 8 A). These bottles are considered most suitable from all standpoints for use in all seasons of the year for germination as well as for the production of seedlings. Although it was found that hyacinth seeds appear to require an after-ripening period of about two months before they will germinate, under optimum conditions 100 per cent germination was obtained eventually. Of the 22 lots of seeds placed under various conditions, 8 lots gave 100 per cent germination.

In practically all tests wet seeds from the second collection germinated sooner and gave a higher percentage germination than dry seeds from the first collection. The average time for wet seeds to start germinating was 10 weeks, and for dry seeds 16 weeks from the time they were collected. Although seeds were removed from the original storage conditions in the laboratory at various time periods ranging from 9 to 79 days for germination tests, the time when germination started in the greenhouse was nearly the same with respect to the collection date. For example, wet seeds stored 9 days in the laboratory started germinating in the greenhouse in 55 days, whereas those stored for 23 days in the laboratory germinated in the greenhouse in 44 days, the total time periods from collection date being 64 and 67 days respectively. Similar results were obtained with seeds removed from air-dry storage, only in this case the total number of days for germination to start, from the time of collection, was 112 and 111 days respectively. The shortest time period in any test for wet seeds to germinate was 57 days and for dry seeds 84 days from the date of collection. Haigh (6) reported a minimum of 27 days for germination to start. The shortest

TABLE XIV

INFLUENCE OF WATER TEMPERATURE ON THE GERMINATION OF HYACINTH SEEDS STORED PREVIOUSLY FROM THE TIME OF COLLECTION IN A WET OR DRY CONDITION BEFORE BEING PLACED UNDER ONE TO TWO INCHES OF WATER IN AN UNSHADED GREENHOUSE ON DECEMBER 15, 1948. PERCENTAGE GERMINATION IN WATER AFTER 55 DAYS

Heat source	No. of seeds	Pre-storage conditions for seeds	Temp. water, ° C.*	Germ., %
Radiant heater**	246	Dry	36	85
	192	Wet	36	92
	100	Wet	27	6
1,000-Watt nitrogen light**	200	Dry	28	18
	161	Wet	28	72
Control	140	Wet	22	0

* Different temperatures according to distance of seed containers from source of heating unit.

** See Table XII (footnotes ** and †) for time of heating and lighting.

time for seeds to germinate after removal from the laboratory to the greenhouse was six days.

The importance of the temperature of the water for germination of hyacinth seed is shown by the results in Table XIV. These results indicate that a relatively high water temperature (28° to 36° C.) is required for germination, although the minimum temperature has not yet been determined. Failure of any seeds to germinate in the laboratory at a temperature of 21° C. may have been due to an insufficiently high temperature, although light also may have been a limiting factor. Haigh (6) also failed to obtain germination in the laboratory in five months at room temperature or in over two months of 45° C. in diffuse light.

TABLE XV

INFLUENCE OF TEMPERATURE AND MEDIUM DURING STORAGE ON SUBSEQUENT GERMINATION OF HYACINTH SEED IN WATER IN PETRI DISHES IN AN UNSHADED GREENHOUSE WITH AIR TEMPERATURE OF 21° TO 27° C. DURING DAY AND 21° C. AT NIGHT

Pre-storage for 69 days		Age of seed from date collected (days)	Germination of 25 seeds after 40 days, %
Temp., ° C.	Storage medium		
22 Lab.	Air-dry	121 121 87	92 96 84
22 Lab.	Water Peat moss	77 77	100 100
10	Water Peat moss	84 84	52* 76*
5	Water Peat moss	94	84 56*
0.5	Water Peat moss	94 94	84 12**
−5	Water Peat moss	94 94	0 0

* Germination in 30 days.
** No development beyond the cotyledon stage.

Low temperature storage ranging from −5° to 22° C. did not appear to shorten the after-ripening period (Table XV). In fact, a slightly lower percentage germination resulted from storage in water at 0.5° and 5° C., and no germination resulted from seeds stored for 69 days at −5° C. In another test the seeds were subjected to the same storage temperatures for one week instead of 69 days. The results were essentially the same except that seeds stored at −5° C. in water and in peat moss gave 50 per cent germination. Presumably hyacinth seeds can withstand a temperature of −5° C. for at least one week but not as long as 10 weeks.

Reports in the literature relating to the germination of hyacinth seed (4, 6, 11, 14) lack agreement. The present results and conclusions are essentially in agreement with those of Haigh (6) with respect to the ready germination of hyacinth seed in shallow water, the importance of water temperature and light, and the conclusion that the possibility of the spread of water hyacinth by seedling plants is a real and permanent one. In addition to the possibility that the lower temperature and reduced light intensity in deep water may limit the production of hyacinth seedlings in nature (6), it might be expected that the reduced oxygen tension under hyacinth mats would also be a limiting factor. However, Haigh (6) could not induce germination by aeration of water held at 45° C. in the laboratory.

The failure of Penfound and Earle (11) to obtain germination under water in the laboratory is understandable on the basis that minimum temperature and light requirements were probably not furnished in their tests, or it is possible the seeds did not remain under water for a long enough period. The relative importance of light, apart from its effect on water temperature, has not been determined. The successive steps whereby the seeds on the bottom of the container germinated and then rose to the surface after roots had formed (Fig. 8 A) have been repeatedly verified and are suggested as the simplest and most effective method for producing hyacinth seedlings, provided proper temperature and light requirements are furnished. About 2,500 seedlings were produced by germinating the seeds under shallow water.

Various methods of scarifying and cutting the seed coats were tested with the idea of hastening germination under laboratory conditions. Removal of the cap from the pointed end of the seed was the only method of scarification or breaking of seed coat which resulted in prompt germination. After removal of the cap, germination occurred within a few days. The cotyledon emerged within a few hours, and the development of the first leaf and roots occurred in a few days' time when the endosperm and seed case remained attached to the cotyledon. Additional attempts to hasten germination by scarification of the seed were abandoned when it was found that removal of the cap caused prompt germination. The delay in germination of intact hyacinth seeds may be associated with a hard seed coat since Crocker (4) obtained germination of green seeds but not mature seeds in the laboratory. However, failure to obtain germination after breaking the seed coats indicates that the delay in germination of intact seeds may not be a simple case of being impermeable to water unless it should be found that the particular methods used for puncturing or breaking the seed coats injured or killed the embryo in all instances.

Growth of hyacinth seedlings. About two weeks after seedlings rose to the surface of the water (Fig. 8 A) they were transferred to soil which was maintained in a saturated condition (Figs. 7 A and 9 C) much the same as described by Haigh (6). Saturation was accomplished by surrounding porous clay pots with tap water or by placing the seedlings on a slanted water-saturated soil surface in a shallow pan as illustrated in Figure 7 A. Immersion or partial immersion of young seedlings retarded or prevented growth (Fig. 7 A), although they survived for many weeks. The approxi-

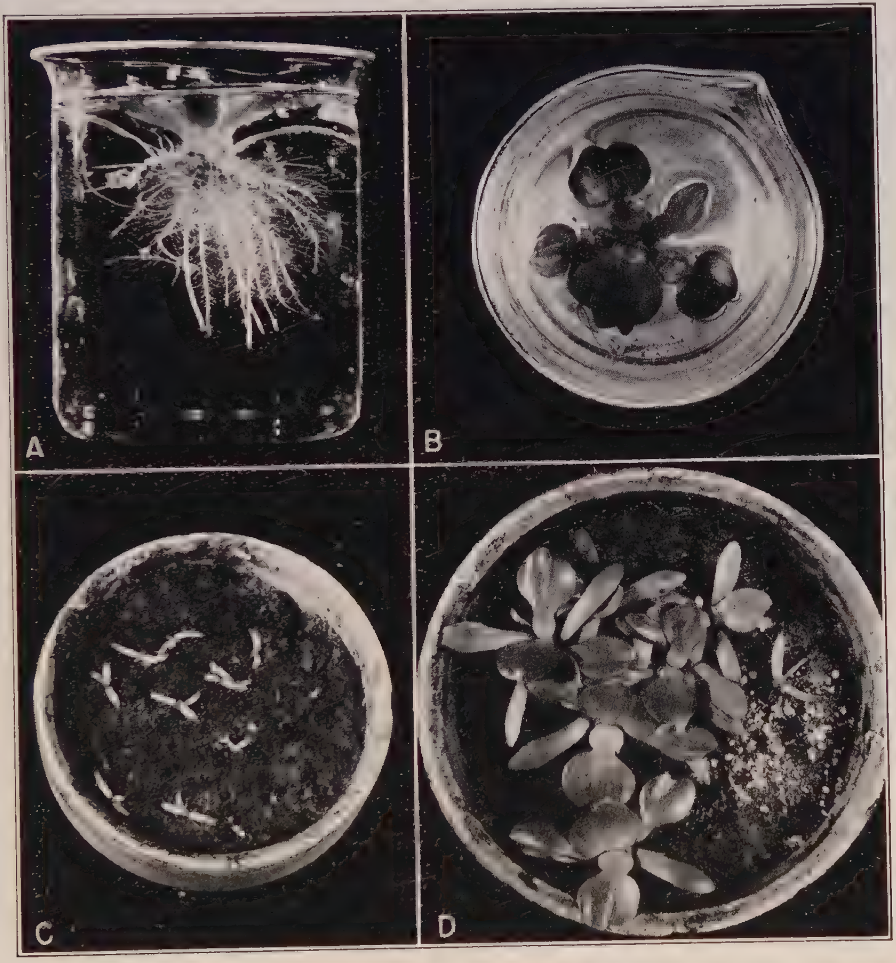

FIGURE 9. A. Seedling hyacinth 56 days after germination showing first crop of float leaves and a root system which developed in water during a 16-day period. B. Same plant viewed from above showing transition from spatulate to float leaves, the latter having bulb-like inflated petioles (this seedling removed from pot shown in Fig. 9 D). C. Young hyacinth seedlings 10 days after planting in wet soil. D. Same seedlings one month later showing transition from ligulate to float-leaved stage at which time they will grow better in water.

mately 700 seedlings shown in Figure 7 A served as a source from which seedlings could be removed at any stage of development to better growing conditions in soil as in Figure 9 C, or in water if the seedlings had developed to the float-leaved stage shown in Figure 9 D.

When the first two or three float leaves were developed to the stage shown in Figure 9 D, the seedlings benefited by transfer to water in contact with a good soil. A seedling with a typical water root system shown in Figure 9 A had grown in water for 16 days after its removal from the pot shown in Figure 9 D. A similar seedling with float leaves was transferred to tap water which was not in contact with soil. During a ten-day period this seedling showed little growth and became chlorotic. Seedlings of the size illustrated in Figure 9 C showed no increase in size for six weeks after a clump of 25 individuals was transferred to water over soil in a container having a capacity of 30 liters. Adult hyacinths in the same container were growing and forming offshoots in a normal manner. Presumably the nutrient requirements for hyacinth seedlings in the ligulate leaf stage are different from those for older plants. The growth and development of young seedlings in our experiments are similar to those described by Penfound and Earle (11).

SUMMARY AND CONCLUSIONS

The 2,4-D spray treatments and methods of applying them to water hyacinths (*Eichhornia crassipes* Solms.) in Louisiana at all seasons of the year, proved substantially more effective and offer greater possibilities for the practical control of this serious pest than treatments or methods reported to date by other workers. Doses of 2,4-D which were effective for killing and sinking hyacinths are considerably higher than those reported by others. The experimental and practical results in controlling water hyacinths by means of 2,4-D were the same for plants in the experimental pits as for plants growing in canals, bayous, borrow pits, and other waterways.

In field tests in Louisiana the alkanolamine salt of 2,4-D killed and sank 90 to 100 per cent of the treated water hyacinths during the period May, 1948, to February, 1949. The principal limiting factors were the concentration of 2,4-D, its rate of application and type of delivery, time of year, the rate of growth and stage of development of the plants, and rain or wave action occurring within three hours after treatment. Effective killing occurred when the concentration of 2,4-D was 0.3 per cent or higher, the rate of application 2 lb. per acre or more, and the rate of delivery 6 to 150 gal. per acre. A higher dose of 2,4-D and a longer time after treatment were required to cause hyacinths to sink than to kill them. A dose ratio of more than two was required to cause significant differences in killing or sinking. The isopropyl and butyl esters of 2,4-D were of about equal effectiveness when compared with the alkanolamine salt. The isopropyl ester and alkanolamine salt of 2,4,5-T were less effective than 2,4-D.

Non-hormone herbicides were ineffective or less effective for killing and sinking hyacinths when used at the approximate minimum effective dose of 2,4-D. None of the non-hormone toxicants, except possibly sodium trichloroacetate, caused additive effects when used in combination with 2,4-D.

With low volume spraying at 25 lb. pressure adequate coverage was not always obtained with the lowest volume nozzles delivering 6 to 18 gal. per acre. Results with the 43 and 57 gal. per acre nozzles were more consistent under all conditions.

The maceration and sinking of hyacinths were presumably associated with the presence of 2,4-D since non-treated plants which died under a canvas shade due to lack of light macerated slowly and did not start to sink until three months after the plants had died. Plants killed with chlorates or arsenicals also macerated and sank more slowly than those killed by 2,4-D.

High pressure spraying with a Bean gun operated at about 400 lb. caused killing and sinking of 80 per cent or more of the hyacinths in borrow pits during October, 1948, and January and February, 1949, when the temperature at the time of treatment ranged from 0° to 27° C. Although hyacinths were killed effectively at all times of the year, the preferred time for spraying is from August through February when reinfestation occurs at a slow rate. Effective killing of hyacinths in borrow pits depended upon an efficient method of spraying which consisted of delivering an effective 2,4-D spray only within the known killing range of the sprayer on a single spray trip with no back ups. For a Bean gun operated at 400 lb. pressure, the killing range was about 25 feet when an effective concentration of 2,4-D was delivered at the rate of about 100 gal. per acre. In this case 2,4-D was applied at the rate of 5 to 10 lb. per acre. Areas greater than 25 feet in diameter were reduced by successive spraying at one- to two-month intervals after the initial treatment.

Improvement of spraying equipment and methods of applying sprays are now being undertaken in order to increase the efficiency of the treatments used for killing hyacinths.

Treatments with 2,4-D at the rate of 2 to 8 lb. per acre killed hyacinths but did not kill alligator weed (*Alternanthera philoxeroides* [Mart.] Griseb.). The isopropyl ester of 2,4,5-T proved effective, in preliminary tests, for killing and sinking alligator weed, although it was relatively ineffective for killing and sinking hyacinths unless used in excess of 8 lb. per acre. Treatment of all alligator weeds in an experimental pit with the trichloro ester, including all plants anchored to the banks, caused killing and eventual sinking of most of the plants. Some regrowth occurred after six months from plants at the shallow end of the pit. In contrast, the alkanolamine salt of 2,4,5-T caused killing but relatively little sinking. However, most of the remaining leafless stems exhibited no growth up to six months after treatment.

Results of germination tests with hyacinth seed were essentially in agreement with those of Haigh. Seeds of water hyacinth collected in October and November, 1948, from submerged seed stalks in the vicinity of Norco, Louisiana, germinated readily in greenhouse tests during December, January, and February at Yonkers, New York. The seeds which were kept wet from the time they were collected and were stored in tap water upon arrival in Yonkers germinated better than seeds which were air-dry.

Hyacinth seeds covered with tap water ranging in depth from a few millimeters in Petri dishes to two inches in wide-mouthed bottles or in evaporating dishes, gave 100 per cent germination when the temperature of the water was 28° to 36° C. during the middle of the day and the containers remained in an unshaded greenhouse with air temperatures of 21° to 27° C. during the day and a night temperature not falling below 21° C. The failure of other workers to obtain germination of intact hyacinth seeds when they were placed on moist media or under water in the laboratory was probably due to an insufficiently high temperature and to lack of sufficient light.

After hyacinth seeds germinated on the bottom of the container, they rose to the surface of the water as seedlings when roots and one or two leaves had developed. Although the idea is prevalent that seedlings rise to the surface of the water after germinating under water, some workers claim that this has never been observed in nature. That hyacinth seeds germinated under water in the greenhouse give rise to normal seedlings may be judged by the fact that about 2,500 seedlings produced by this method are now growing in soil and water in all stages of development from newly germinated seeds to seedlings which have developed float leaves and offshoots. The maximum depth of water under which hyacinth seeds will germinate has not been determined.

Germination of intact wet hyacinth seeds started about two months after they were collected, while dry seeds took nearly twice as long, regardless of various conditions to which they were subjected before being placed in the greenhouse. Hyacinth seeds withstood storage at −5° C. for one week (50 per cent germination) but not for ten weeks, since in the latter case no germination had occurred at the end of seven weeks after removal from storage. In view of the results showing that hyacinth seeds have the capacity to germinate under shallow water and to tolerate −5° C. for one week, the potential possibilities for the propagation of hyacinths by seed are tremendous. This is contrary to the views of some workers. The principal factors which limit the production of seedling hyacinths in nature are believed to be temperature and light.

Up to the stage where the first float leaves are formed, hyacinth seedlings grew best under greenhouse conditions in soil saturated with water, and thereafter in water containing some of the nutrients normally present in a good soil. A composted soil added at the rate of 100 to 150 grams per liter of tap water proved satisfactory for the growth of seedlings with the first crop of float leaves and for the growth and production of offshoots on adult plants for a period of two months or longer. Flowering of adult plants occurred when the night temperature did not fall below 21° C. but failed to occur when the night temperature fell to 16° C. each night, even though normal light was supplemented with six hours of artificial light between 6 p.m. and midnight.

ACKNOWLEDGMENT

The authors wish to acknowledge the cooperation of Colonel Hardin's staff, especially the helpful suggestions given by Mr. Walter Carey and the cooperation of Mr. C. R. Dunn and Mr. C. Tomlinson who helped design and maintain spray equipment and man the application of winter sprays.

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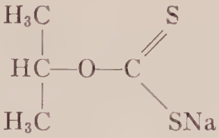
SODIUM ISOPROPYLXANTHATE AS A NEW SELECTIVE HERBICIDE

Luther L. Baumgartner[1] and Benjamin Wolf[2]

Chemical weeding of vegetable or grain crops after a weed problem presents itself is generally more economical than mechanical weeding if satisfactory selectivity is obtainable. This paper is concerned with the presentation of data indicating that sodium isopropylxanthate (hereinafter referred to as n.i.x.) will selectively weed lima beans (*Phaseolus limensis* Macf., var. Ben Fish Thorogreen), peas (*Pisum* sp., var. Glacier), and cabbage (*Brassica oleracea* var. *capitata* L.).

DESCRIPTION OF THE CHEMICAL

N.i.x. is a water soluble compound that can be applied as a concentrated spray, dilute spray, or dust. Its high water solubility (30 per cent at 4° C., 46 per cent at 24° C., and 54 per cent at 35° C.) enhances its activity as a dust and reduces the possibility of toxic residues being left in the soil. To date, no residual toxicity has been noted. Its chemical formula is as follows:



METHODS

All applications of n.i.x. were made with knapsack sprayers or hand dusters, except for one application on lima beans, in which case a power sprayer was used.

The study plots were generally 100 square feet in size, and the predominant weeds existing therein were annuals. With the exception of one large-scale test, no cultivation was practiced in these plots.

The effectiveness of the herbicide was determined by estimating the number and/or weighing sample quantities of weeds of each kind remaining after treatment, and comparing with the quantity of weeds in the untreated plots. All weed weights were wet weights. Weed weight samples were obtained from five one-square-foot blocks selected at random from each plot. One exception to this practice is indicated in Table I, in which case all the weeds were weighed. The minimum number of replicated plots was two, and the maximum was four.

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The experiment related to weed control in cabbage was conducted at Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York, and the remainder at Seabrook Farms, Bridgeton, New Jersey. Weeds common to these plots were as follows: crabgrass (*Digitaria* spp.), yellow foxtail (*Setaria glauca* [L.] Beauv.), ryegrass (*Lolium* sp.), clear weed (*Pilea pumila* [L.] Gray), smartweed (*Polygonum* spp.), lamb's quarters (*Chenopodium album* L.), pigweed (*Amaranthus* spp.), common chickweed (*Stellaria media* [L.] Cyrill.), mouse ear chickweed (*Cerastium vulgatum* L.), purslane (*Portulaca* spp.), spotted spurge (*Euphorbia maculata* L.), evening primrose (*Oenothera biennis* L.), milkweed (*Asclepias* spp.), bindweed (*Convolvulus* spp.) seedlings, henbit (*Lamium amplexicaule* L.), purslane speedwell (*Veronica peregrina* L.), bedstraw (*Galium* spp.), fleabane (*Erigeron* spp.), ragweed (*Ambrosia* spp.), Spanish needles (*Bidens* spp.), and galinsoga (*Galinsoga parviflora* Cav.).

TABLE I

WEED CONTROL IN CABBAGE WITH SODIUM ISOPROPYLXANTHATE

Rate of application, lb./acre	Wet wt. of weeds, g./sq. ft.	Estimated quantity and kind of remaining weeds in plots, % of total						
		Galinsoga	Purslane	Crabgrass	Lamb's quarters	Chickweed	Spanish needles	Miscellaneous
155.7	4.5	0	0	100	0	0	0	0
83.1	18	0	0	95	0	0	0	5
34.6	35	0	0	95	0	0	0	5
17.3	27	0	40	30	10	10	10	0
0	477	50	40	0	0	0	0	10

PRESENTATION AND DISCUSSION OF DATA

To control annual garden weeds in a cabbage planting a dust consisting of n.i.x. diluted with Pyrax was spread between the rows and at the bases of plants planted two days earlier. It was applied at rates of 17.3, 34.6, 83.1, and 155.7 pounds per acre. The plots varied in size from 92 to 138 square feet. The dust was applied August 19, 1947, and final observations were made October 6, 1947, when the cabbage was in head.

No injury to cabbage resulted from these applications. Repetition of this experiment during 1948, using 17 pounds per acre of n.i.x. in a water spray, caused no injury, even though the herbicide was sprayed over the cabbage at the same rate it was applied between the rows.

The weeds commonly growing in the vicinity of these plots were galinsoga, purslane, crabgrass, lamb's quarters, chickweed, Spanish needles, and smartweed.

It will be noted in Table I that all rates of application resulted in considerable control of weeds, as compared to the untreated plot, but a greater variety of weeds emerged in the 17.3 pounds per acre plot. It is important to observe that only crabgrass consistently occurred on all plots except the untreated one. This is readily explained by the fact that the growth of galinsoga and purslane was extremely dense. From these data and common conceptions of commercial weed control, it would appear that there is no important difference between the rates of 17.3 and 155.7 pounds per acre when compared with the untreated plot.

TABLE II

COMPARISON OF WEED CONTROL IN GLACIER PEAS BETWEEN DUST AND WATER DILUENTS

Treatment	Application of n.i.x., lb./acre	Av. wet wt. of weeds, g./sq. ft.	Estimated quantity and kind of remaining weeds in plot, % of total								
			Lamb's quarters	Chickweed	Pigweed	Henbit	Crabgrass	Ryegrass	Miscellaneous weeds	Miscellaneous grasses	Miscellaneous annuals
Water spray (100 gal./acre)	30	5.6	0	1	4	0	90	5	0	0	0
	25	5.6	0	0	0	35	60	0	5	0	0
	20	4.6	5	0	0	20	70	0	5	0	0
	15	6.2	5	0	0	5	85	0	5	0	0
	10	4.6	0	0	0	50	50	0	0	0	0
	0	67.6	40	45	3	10	1	1	0	0	0
Dust (diluent added to make 75 lb./acre)	30	3.6	0	0	0	0	85	0	0	15	0
	25	2.2	0	0	0	0	95	0	0	5	0
	20	1.8	0	0	0	0	90	0	10	0	0
	15	5.8	0	0	0	0	85	10	0	0	5
	10	5.4	0	0	0	5	55	30	0	0	10
	0	81.2	35	40	5	0	15	0	5	0	0

In another series of experiments (data from one of the series are given in Table II) n.i.x. was applied to peas (var. Glacier) at a time when they were about 7 inches high, and the weeds were from 0.5 to 2 inches high. It was applied as a spray, using 100 gallons of water per acre, as a diluent, and as a dust, diluted with enough Homer clay to make an application rate of 75 pounds per acre. The water spray application was made April 29, 1948, and the dust applications May 4, 1948. Final observations were made June 9, 1948. During this period, these plots received 10.41 inches of rain, and were inundated at least twice.

It will be noted in Table II that there was very little difference in weed control between the different rates. The smaller amount of crabgrass in the untreated plots and those receiving only 10 pounds per acre is, again, believed to be more closely related to the influence of competing weeds than to the effect of the herbicide.

Although weed control was obtained with all applications, rates greater than 15 pounds per acre caused injury to the basal leaves of the peas.

TABLE III

COMPARISON OF WEED CONTROL OF DILUTE SPRAYS COMPARED WITH CONCENTRATED SPRAYS

Application/acre		Wet wt. of weeds, g./sq. ft.	Av. no. of each kind of weed in plots						
Gallons of H_2O	Lbs. n.i.x.		Crabgrass	Pigweed	Purslane	Bedstraw	Lamb's quarters	Yellow foxtail	Ragweed
75	60	9.0	38	2	2	3	1	0	0
	30	44.0	60	1	3	1	1	0	0
	15	104.6	107	2	0	9	0	0	0
50	60	12.0	28	0	0	2	0	0	0
	30	20.0	51	1	3	6	1	0	0
	15	50.8	39	1	4	5	1	20	0
25	60	17.8	23	1	0	2	0	0	0
	30	10.6	24	0	1	10	0	11	0
	15	69.0	35	1	0	10	0	30	0
Untreated		186.2	80	2	20	78	20	36	3

To determine the herbicidal activity of n.i.x. when applied before the weeds emerge, a series of bare plots were prepared by scraping and cultivation. Crabgrass and pigweed germinated so rapidly, however, that some of these were beginning to emerge at the time of application. The applications were made on June 17, 1948, and final observations made on July 20, 1948. During this period, these plots received a total of 3.91 inches of rain.

On the assumption that more material would be required for pre-emergence applications than for post-emergence applications, the range of rates extended from 5 to 60 pounds per acre. Table III, however, contains only data for the 60, 30, and 15 pounds per acre rates, because the 10 pounds per acre plots were destroyed, and the weed control on the 5 and

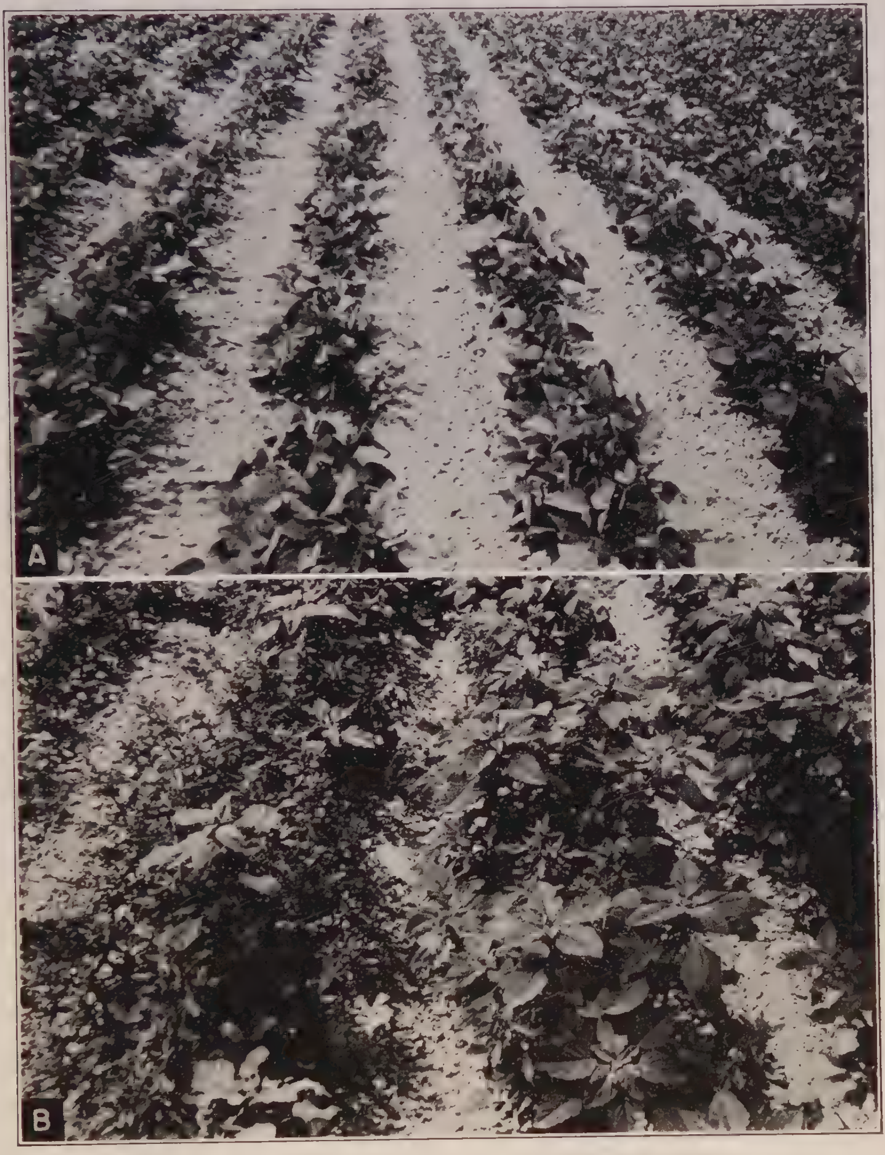

FIGURE 1. Weed control in lima beans with sodium isopropylxanthate. A. An application of 12 lb./acre was made just previous to opening of trifoliate leaves. B. No treatment. Photograph taken as flowers were beginning to open. The untreated area had been cultivated three times and the treated area once.

7.5 pounds per acre plots was essentially negative. The lack of adequate nozzles prohibited these applications from being made at a rate of less than 25 gallons per acre. Here again it will be noted that considerable control of annual weeds has been obtained, except for crabgrass.

Weed control that might be obtained under ideal conditions was represented in an experiment designed to weed selectively small-seeded lima beans (Fig. 1). This acre plot of beans was sprayed with n.i.x., using 50 gallons of water containing 12 pounds of n.i.x. per acre. The trifoliate leaves had not yet expanded, and the weeds were beginning to appear above the ground. No precipitation occurred immediately following this application, and a dry period occurred near harvest, when a second germination of weeds would normally occur. The application was made June 22, 1948, and final observations were made July 20, 1948. A total of 3.22 inches of rain occurred during the first two weeks of this period.

For water conservation and maintenance of desired soil texture, it was desirable to cultivate this plot at least once. The untreated area, however, required three cultivations. Furthermore, hand weeding was necessary to save the bean crop in the untreated area.

The degree of weed control is illustrated by the following data, which were taken from ten strips, each ten feet long, and with a width consisting of three inches on either side of the drill row, selected at random. Thus, each sample area consisted of five square feet. The samples were taken along the drill rows because the serious weed problem occurred in this strip, inasmuch as cultivation adequately controlled weeds between the rows. The treated rows contained 30 g. of weeds per 100 linear feet (wet weight) of bean row, as compared with 2,230 g. per 100 linear feet in the untreated row. The weeds in the former plots were crabgrass and yellow foxtail, but the untreated rows contained approximately 80 per cent pigweed, 10 per cent lamb's quarters, 5 per cent purslane, and 5 per cent miscellaneous grasses.

An experiment designed to compare the herbicidal activity of n.i.x. without the addition of a wetting agent, as in the foregoing experiments, with its activity in conjunction with a wetting agent, consisted of six rows of semi-mature potatoes (*Solanum tuberosum* L.) and associated garden weeds. The potato vines were not killed when sprayed with n.i.x. alone at concentrations of 0.5, 1.0, and 1.5 per cent, but the 1.0 and 1.5 per cent concentrations gave complete kill when admixed with 0.125 per cent sodium lauryl sulfate. The effect on the weeds, principally six- to eight-inch purslane and lamb's quarters, was similar.

Although n.i.x. can be applied more uniformly as a spray, it appears to be just as effective when applied as a dust, except that slightly more n.i.x. may be required to assure good coverage when a dust is used. Variations in rainfall and temperature may affect its activity. During the course of the experiments, it was observed that weeds died more rapidly when applications were made during periods of high temperature, although hot weather following application did not appear to influence its activity.

SUMMARY

Data on the use of sodium isopropylxanthate as a new selective weed killer are presented. It controlled annual weeds such as purslane, pigweed, bedstraw, lamb's quarters, henbit, and ragweed, in lima beans, peas, and cabbage. Application of this herbicide at rates of 10 to 12 pounds per acre selectively killed annual weeds in peas and beans, without causing injury to the crops. Rates above 15 pounds per acre caused injury to peas. Cabbage was not seriously injured when sprayed at the rate of 17 pounds per acre.

This herbicide, possessing high water solubility, can be applied as a dust or liquid spray.

ZINC DIMETHYLDITHIOCARBAMATE-CYCLOHEXYLAMINE COMPLEX[1] AS A DEER REPELLENT APPLICABLE TO AGRICULTURAL CROPS

Luther L. Baumgartner[2] and Stephen E. Powell[3]

THE PROBLEM

Coincident with the return of huntable deer (*Odocoileus virginianus* Merriam) populations in the United States came the problem of deterring them from agricultural crops. The actual amount of deer damage is reportedly extensive and acute. Damage claims and adjustments for the northern tier of States are estimated to range from $150,000 to $500,000 per state per year (correspondence from seven State Conservation Commissions). Consequently, one of the most important problems facing agriculturalists and game managers today is the development of an effective deer repellent. This paper describes the effectiveness of a chemical, zinc dimethyldithiocarbamate-cyclohexylamine complex (hereinafter called z.a.c.), for this purpose.

EARLY ATTEMPTS TO REPEL DEER

Early attempts to repel deer were generally related to mechanical devices, often representing engineering genius, but having little deterrent value. The devices were usually variations of automatically or manually operated guns, flashing lights, mirrors, noise makers, and scare-crows. In almost every case, deer soon lost their fear of them and continued their browsing of crops. Early reports of the effectiveness of many of these deer-damage control methods were made by Adock (1), Barrett (2), Burr (3), Dale (4), Gerstell (5), McAtee (6), Middour and Morton (7), Mills (8), and Weiss (9).

Electric fences of various types were tried, but only one, consisting of a four- to four and one-half-foot barrier with an outrigger supporting a fine electrically-charged wire about 30 inches from the fence and about 15 inches above the ground, has been effective, but then only for certain seasons. Electric fences do not operate under all conditions in winter, and they can easily be shorted by grass, brush, or objects falling against them.

Ordinary fence barriers may be effective if maintained in perfect condition and built six to eight feet high. Under certain conditions, two or three strands of barbed wire attached to two-foot outriggers nailed to the tops of fence posts are effective, but, in general, their use is limited to special purposes. The most important objection to fence barriers is their high cost of construction and maintenance.

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[1] Now formulated with an adhesive by The B. F. Goodrich Chemical Company, Cleveland, Ohio, under the trade name of Good-rite z.i.p.

[2] The B. F. Goodrich Biochemical Laboratory at Boyce Thompson Institute for Plant Research, Inc., Yonkers 3, New York.

[3] Maine Department of Inland Fisheries and Game, Augusta, Maine.

Chemicals such as lime sulfur, blood albumin, creosote, and oil of anise had been tried for many years, but it was only after World War II that intensive studies of chemical animal repellents were undertaken. Although several organizations were engaged in this new approach, the bulk of these studies was conducted by the United States Fish and Wildlife Service and the Maine Department of Inland Fisheries and Game.

Because deer eat almost every agricultural crop, ranging from strawberries (*Fragaria* sp.) to fruit trees, an effective repellent must exhibit wide latitude in method of application. It seems improbable that one repellent will deter deer under all circumstances; however, z.a.c. has been effective in materially reducing deer damage in many crops.

DESCRIPTION OF CHEMICAL AND ITS MODE OF ACTIVITY

Z.a.c. is an organic chemical complex with very low water solubility. It is essentially tasteless but has slight odor to humans, and may cause nasal irritation to some persons. Present information indicates that it is harmless to animals, and that it is non-toxic to plants at the dosage used.

It is necessary that deer ingest a portion of the plants treated with this chemical before being deterred. Deer observed usually ate from three to five bites of snapbeans (*Phaseolus* sp.), and then spit the next bite out and left the area.

METHODS

In 1947 a cooperative effort between the Maine Department of Inland Fisheries and Game and The B. F. Goodrich Biochemical Laboratory was begun to extend the then-existing deer repellent studies. The former provided a testing area well populated with deer and the necessary personnel to make specific applications, and the B. F. Goodrich Biochemical Laboratory supplied chemicals to be tested and technical assistance.

The preliminary testing area was Swan Island, an island of 1,300 acres in the Kennebec River near Richmond, Maine, which supported a concentrated population of approximately 200 deer. As many as 81 deer have been counted at one time browsing in the vicinity of the test plots.

About 50 per cent of Swan Island is covered by a partially cut-over pine-beech-maple forest. The remaining portion is almost equally divided between cultivated and abandoned cultivated land. Most of the intensive studies were conducted on the island, while the extensive tests were conducted on mainland farms within a radius of 65 miles from Richmond.

The repellent was applied as a dust and as a water spray, but the effectiveness of either method was temporary unless an adhesive was added.

Consequently, best applications were mixtures of z.a.c. with enough adhesive to hold it fast. The adhesive used was polyethylene polysulfide, a rubber-like material commercially available as Good-rite p.e.p.s. Deposits of this formulation remained on leaves for several months.

DISCUSSION OF EXPERIMENTAL RESULTS

The first formulation of z.a.c. was tested by Mr. Nathan W. Fellows, Jr., of the Maine Department of Inland Fisheries and Game, during the winter of 1947 on one-pound samples of cedar (*Thuja occidentalis* L.) browse. This formulation was effective as a repellent, but lacked weathering ability.

An improved formulation of it was tried qualitatively on buckwheat (*Fagopyrum esculentum* Moench) and snapbeans during the summer of 1947, and protection was obtained for the portion of the plants covered. In these tests, the repellent was applied by first spraying the plants with the sticker and then dusting them with z.a.c. before the sticker dried.

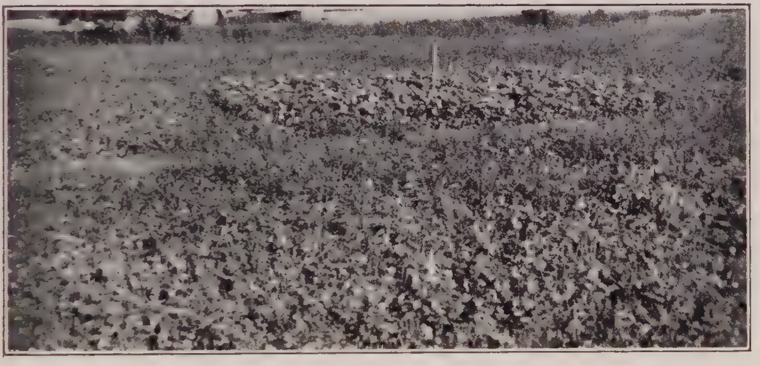

FIGURE 1. A 100-square-foot plot of buckwheat treated with the repellent. The buckwheat in the surrounding portion of the field was completely browsed.

The first buckwheat plot, blocked off in a field, contained approximately 100 square feet of 12-inch high buckwheat in which deer had already begun to browse (Fig. 1). The repellent was applied on June 26th, and the plants remained untouched for nine days, while the surrounding buckwheat was eaten to ground level in two days. Deer began feeding on the "island" of treated buckwheat on the tenth day, and continued to consume small amounts until a large part of it was consumed 31 days after the application. This feeding was limited almost entirely to the new growth which developed after the tenth day. Furthermore, there was some indication, based on observations, that different groups of deer tested the treated buckwheat on different nights, which accounted for some of the treated leaves being eaten.

Closer observations on another test containing 200 square feet of buckwheat illustrated, in part, how deer reacted to this repellent. This plot was so located that some deer would pass through it in order to reach the untreated buckwheat. Deer passed through this area periodically for 13 days without browsing more than an occasional sample leaf. However, by the 14th and 15th days, sufficient new growth had developed on the treated plants to again be attractive to deer, and within two nights the plot was browsed down to the originally sprayed leaves. The adjacent untreated buckwheat was completely eaten in two days.

In a third test, the repellent was applied to three rows of snapbeans, each 100 feet long. The deer were deterred for 16 days, while the adjacent untreated beans were completely eaten at the end of five days.

This formulation was again tested under winter conditions, beginning February 5th, in a deer yard on Swan Island. It was formulated as a water dispersion of 5 per cent z.a.c. and 1 per cent adhesive. A half-ton truckload of cedar cuttings was sprayed with a pressure sprayer and placed in two piles at the edge of the deer yard. Adjacent were two similar piles of untreated browse. Ten hours later (overnight) the untreated piles were completely eaten, but no browsing was observed on the treated piles. Five days later, the check piles were replaced with fresh cedar, and these also were eaten in one night. The treated piles remained untouched for ten days. On the 11th night, approximately 2 per cent was eaten, and small amounts continued to be eaten thereafter until it was almost completely consumed, 15 days after having been placed in the yard.

During the 1948 growing season, both quantitative and extensive qualitative tests were made. The quantitative tests were limited to snapbeans. Plots 1, 2, and 3 were prepared on Swan Island, where the deer population was extremely high, but Plot 4 was prepared in a commercial bean field, where the deer population approached a more normal level.

The reaction of the deer herd in the two areas was markedly different. For example, the plots on Swan Island were visited by a large number of different deer, and by the time each deer or group of deer had sampled enough plants (four to six by count) to find the repellent objectionable, considerable damage had resulted. Plot 4, however, was visited nightly by approximately the same few deer, and considerably fewer treated plants were damaged before protection was obtained.

Plot 1 (Figs. 2 A, B and 3 A) consisted of 14 rows, each 200 feet long, of snapbeans. Two outside rows on each side and the center row were untreated. To one side of the center row were four rows sprayed with a 5 per cent dispersion of z.a.c. mixed with 0.5 per cent adhesive. On the other side of the center row were five rows treated with a 3 per cent dispersion of the repellent mixed with 0.5 per cent of the adhesive.

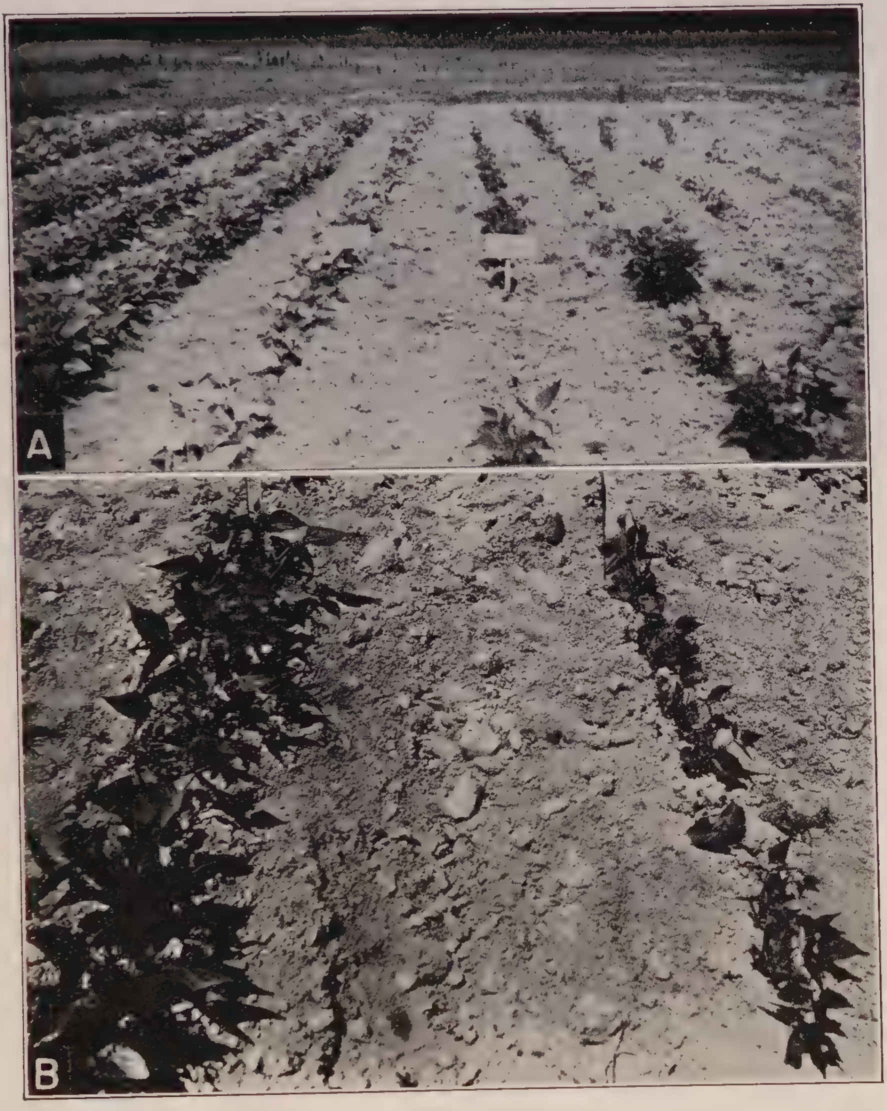

FIGURE 2. A and B. Amount of deer damage to snapbeans after one visit from deer. Left, treated with 3 per cent z.a.c. Right, no treatment.

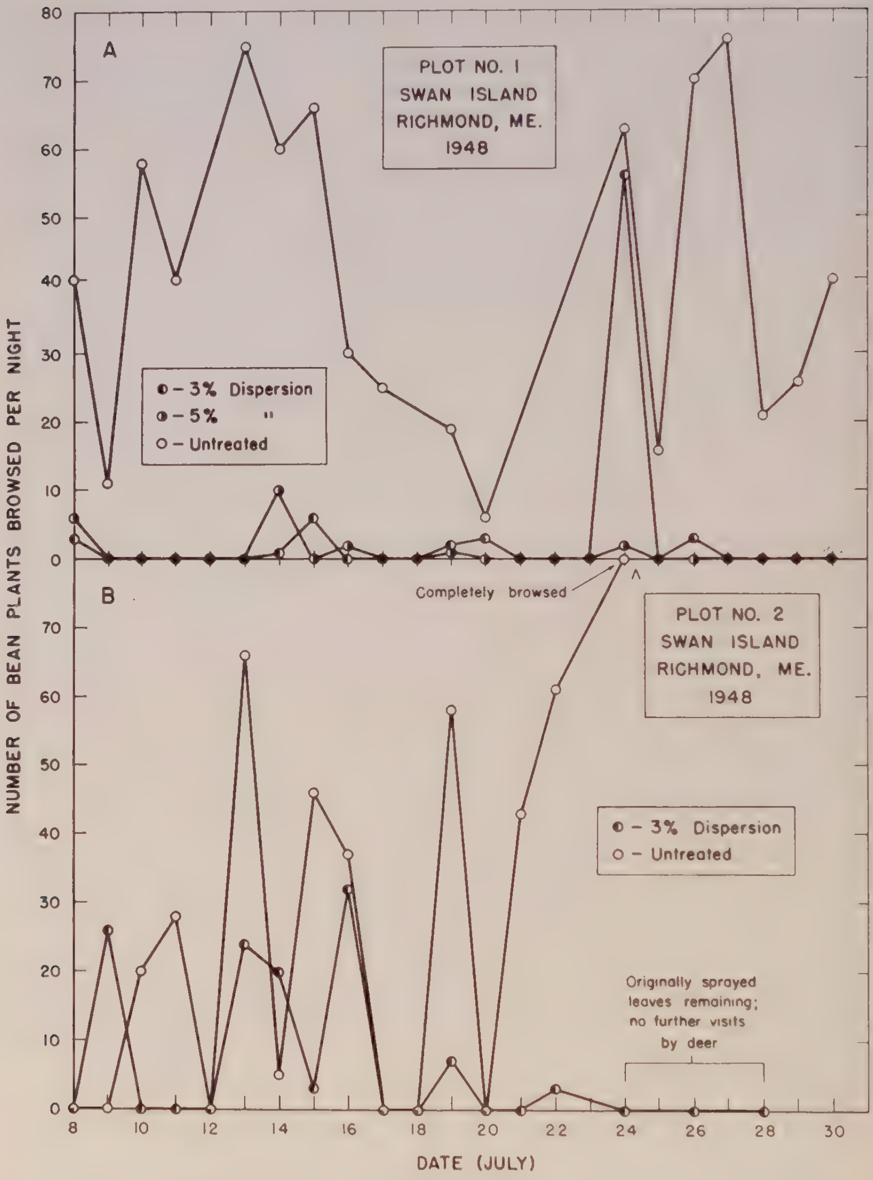

FIGURE 3. Amount of deer damage incurred each night to snapbeans treated with the repellent: (A) 3 and 5 per cent sprays applied July 8, 16, and 24; (B) 3 per cent sprays applied July 8, 16, and 24.

All browsed plants were removed before the spray was applied, and counts were made on each day thereafter, when each newly browsed plant was removed, regardless of amount browsed. Thus, a treated plant with one or two leaves missing was considered as much damaged as an untreated plant eaten to the ground. The apparent lack of protection provided by the 3 per cent application on July 24th (Fig. 3 A) is explained by the fact that there were relatively few untreated plants left, and the untreated new growth on the treated rows offered a better meal. There is no explanation as to why the damage was limited to the 3 per cent treatment, unless one might postulate that the deer entered the field from that side.

Plot 2 (Fig. 3 B) was established to test the effectiveness of a 3 per cent dispersion with the adhesive, and was arranged so that the six treated rows were alternately separated by five untreated rows. Each of the eleven rows was 90 feet long. As many as 60 deer could be seen near this plot at one time, and a great number of deer sampled the beans during the early part of the test, which explains the high rate of damage to treated plants early in the test. Approximately one-half of the treated plants damaged had only one or two leaves missing.

In Plot 3 (Fig. 4 A) a 1 per cent dispersion of the repellent mixed with 0.25 per cent of the sticker was sprayed on seven 100-foot rows, separated in a regular pattern by five untreated rows. Although good repellency is indicated by the data, actually the deer visitations were so irregular that satisfactory interpretation is difficult. Only a few deer visited the area until after considerable new growth was present. As a result, when the deer did visit the plot, they ate almost all the untreated and a part of almost all the treated plants. However, farmer reports on qualitative tests conducted off the island indicated that under many conditions a 1 per cent concentration may be adequate.

Plot 4 (Fig. 4 B) consisted of a one-acre plot at the corner of a 20-acre bean field. Two-thirds of the plot was surrounded by woodland, and was chosen as a desirable plot because deer had already eaten several hundred bean plants in one corner of the field. The other 19 acres were protected with an electric fence.

There were four 350-foot rows in each block of the 4 per cent and 5 per cent treatments, each of which was replicated twice. Equal-sized blocks of the 3 per cent treatment were, however, replicated three times. No untreated rows could be left, but each morning the deer tracks of the previous day were erased after a study of the tracks was made to determine the number of deer present during the past 24 hours. Deer were feeding regularly in the adjacent untreated portion of the bean field, until an electric fence was erected around it two days after the application of the spray. Moose (*Alceus americana* Clinton) visited the area three times and sampled the treated beans, but caused less damage than the deer.

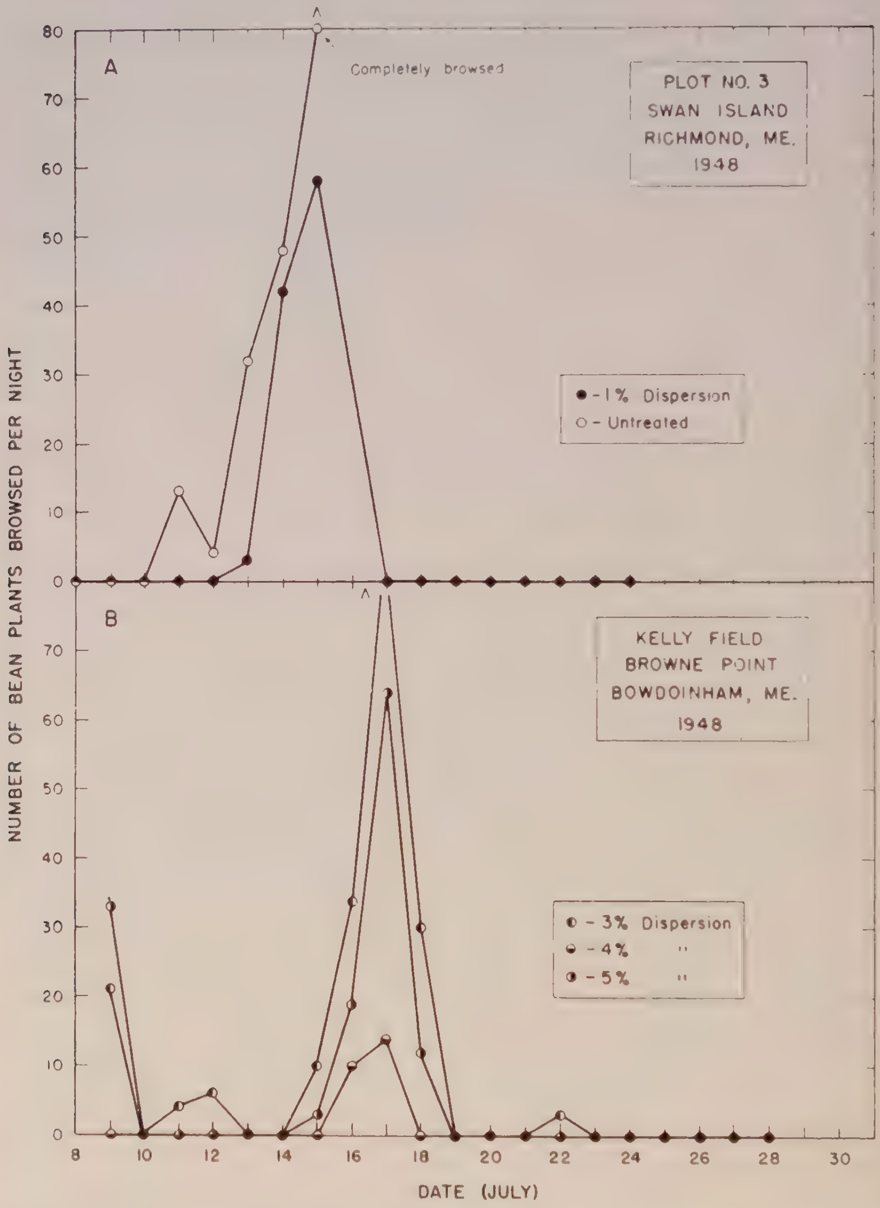

FIGURE 4. Amount of deer damage incurred each night to snapbeans treated with the repellent: (A) 3 per cent spray applied July 8 and 16; (B) 3, 4, and 5 per cent sprays applied July 9, and 3 per cent spray applied on entire area July 17.

The initial damage in the first five days of growth before treatment, as determined by sampling a portion of the total number of bean stalks in the plot, was 1.2 browsed stalks to each linear foot. The sample consisted of 20 per cent of each third row, totaling 270 feet of beans. The amount of damage incurred by deer during the 21 days following application of the repellent was one plant in 27 feet. Most of this occurred on the 16th and 17th of July (Fig. 4 B). In actual practice, most of this damage would have been avoided by keeping the new growth reasonably covered. Had this not been an experimental plot, the second application would have been made two days earlier.

The extensive tests were conducted on farms whose owners requested the Department of Inland Fisheries and Game to protect their crops from deer. The reports secured from these farmers regarding the effectiveness of this repellent are considered to be significant and to carry a minimum of bias. These applications were made on 29.6 acres of beans on eleven farms, 4.5 acres of strawberries on three farms, 3,630 apple (*Malus* sp.) trees in five orchards, plus an uncounted number of small plots of miscellaneous vegetables and flowers. Approximately 50 per cent of these applications were made with hand sprayers and the remainder with power sprayers, but no difference was observed between the two methods of application.

All but two of the farmers reported good to complete protection from deer using from two to three applications of the repellent, depending upon the crop and rate of new-growth development. One exception was a farmer who sprayed a 20-foot band around the edge of his bean field. The beans in the sprayed border were protected, but the deer ate the beans inside it, and the farmer concluded that z.a.c. was ineffective.

In another case, the repellent was applied on peas (*Pisum* sp.), but did not adhere to them. The ultimate result was that the deer passed over all the other crops in the garden and ate most of the peas. This is a problem in formulation, and can readily be solved.

It was not possible to secure yield data on all the extensive tests, but in one instance a treated bean field was reported to have yielded 14 bushels of beans per acre, while the yield of an immediately adjacent untreated portion of the field was but 7 bushels per acre. In another case, the treated field yielded 18 bushels per acre, while untreated portions produced but 4 bushels per acre. The current value of the beans was $15 per bushel. The cost of the treatment is estimated to be less than $7 per acre.

SUMMARY

A new deer repellent, zinc dimethyldithiocarbamate-cyclohexylamine complex, was found to be unpalatable to deer. Vegetable crops, normally susceptible to serious deer damage, could be grown to maturity in localities having high deer populations. Crops such as snapbeans, strawberries, buck-

wheat, and fruit trees have been protected from deer with applications of from 1 per cent to 5 per cent concentrations of the spray. Deer were deterred only from food treated with the repellent, consequently it was necessary to make repeated applications, depending upon rate of new growth development.

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FORMATIVE EFFECTS OF SEVERAL SUBSTITUTED PHENOXY ACIDS APPLIED TO KALANCHOE

P. W. Zimmerman and A. E. Hitchcock

Formative effects induced by hormone-like chemical compounds and the resulting modification of organs of plants have received considerable attention since the first publication on substituted phenoxyaliphatic acids and substituted benzoic acids appeared (6). Various species of plants differ in their susceptibility to different compounds. In some cases the species may respond to the acetic acid form but not to the higher homologs (2, 4, 5).

Kalanchoe daigremontiana Hamet et Perrier, a succulent plant, is particularly sensitive to some of the substituted phenoxy acids (1, 3). Odd-looking monstrosities have been made by the application of 4-chlorophenoxyacetic acid.

The purpose of this report is to record some observations made since 1942 when the work was started.

While several methods have been used, the chemical was generally applied as a lanolin preparation. This consisted of mixing from 1 to 20 mg. of the chemical in 1 g. of lanolin and applying a small amount with a glass rod to the uppermost node of the stem. Concentrations most frequently used were 0.25, 0.50, and 1.0 per cent. The response was more pronounced when the preparation was applied to young than to old tissue.

The chemical compounds used were principally substituted phenoxyacetic, propionic, and butyric acids, and substituted benzoic acids. These were prepared for the most part in the Boyce Thompson Institute laboratories during 1942 and 1943. In 1948 Dr. J. M. F. Leaper of the American Chemical Paint Co., Ambler, Pa., supplied a number of substituted phenoxy acids which were checked with some of those made in our laboratory. Specific compounds are listed with the report of results.

The most pronounced formative effects were caused by 4-chlorophenoxyacetic acid applied to the youngest internode of the main stem. The response varied with the concentration of the chemical in the lanolin preparations, but a range of 0.25 per cent to 1 per cent was found satisfactory for causing all of the different effects.

The first noticeable response was hyponasty of the young leaves. This occurred in the course of a few hours and was pronounced in 48 hours. Some substituted phenoxy compounds caused epinasty, while others had no effect. The different bending responses are illustrated in Figure 1. Of special interest is the fact that 4-chlorophenoxyacetic acid and α-(4-chlorophenoxy)propionic acid caused hyponasty while the higher homolog,

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α-(4-chlorophenoxy)butyric acid, did not. As a contrast, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid caused epinasty of young leaves. 2,3,5-Triiodobenzoic acid also caused hyponasty of *Kalanchoe* leaves. 3-Chlorophenoxyacetic acid did not induce hyponasty but had a tendency to induce adventitious roots to grow where the chemical was applied.

A complete survey of substituted phenoxy and benzoic acids for the capacity to induce hyponasty or epinasty has not been made. With the information at hand no reason can be assigned for the variation in response caused by closely-related compounds.

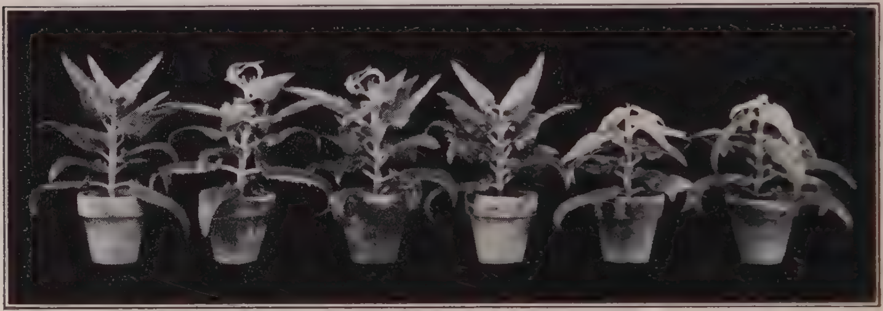

FIGURE 1. *Kalanchoe* plants showing responses to substituted phenoxy acids in lanolin preparations, 5 mg. per g., except 2,4-dichlorophenoxyacetic acid which was applied at 1 mg. per g. Left to right: Control showing normal growth habit; 4-chlorophenoxyacetic acid causing induced hyponasty; α-(4-chlorophenoxy)propionic acid causing hyponasty; α-(4-chlorophenoxy)butyric acid like control; 2,4-dichlorophenoxyacetic acid causing epinasty; 2,4,5-trichlorophenoxyacetic acid causing epinasty.

Variation in response according to the location of the substituted elements was evident when the 2, 3, and 4 positions on the benzene ring were involved. The compound with a halogen in the 2 position caused only swelling of the treated stem tissue. Substituted in the 3 position, the compound had a tendency to induce roots around the stem where the preparation was applied. However, 4-chlorophenoxyacetic acid caused hyponasty of leaves and considerable swelling or proliferation of the treated tissue followed by pronounced modification of all parts which grew thereafter. Also the proliferating tissues produced adventitious buds. There was considerable variation in the appearance of plants treated alike, but all had a tendency to produce monstrosities. Several monstrosities are illustrated in Figures 2 and 3. Figure 2 A shows a plant which produced a tubular fasciated top with the terminal bud embedded so that it could not function. Leaves and axillary shoots were also tubular. The serrate edge of the leaves characteristic of controls did not appear or was intermingled with smooth edges in the treated plants. Plantlets frequently appeared at odd places on modified leaves. Figure 2 B illustrates plantlets produced and growing on the roughened flat side of fasciated leaves. Since the growing point was embedded it lost its natural apical dominance, and plantlets grew while attached to the leaves. Control plants held the plantlets in check while attached to the parent plant. The chemical influence on treated plants was extended to the plantlets on the leaves as shown by the fact that they also showed a tendency to become tubular. When the plants were treated they always had some partially developed leaves. These went ahead to develop but showed some of the peculiar effects of the chemical. For example, the leaves became tubular, and plantlets were released from natural dominance.

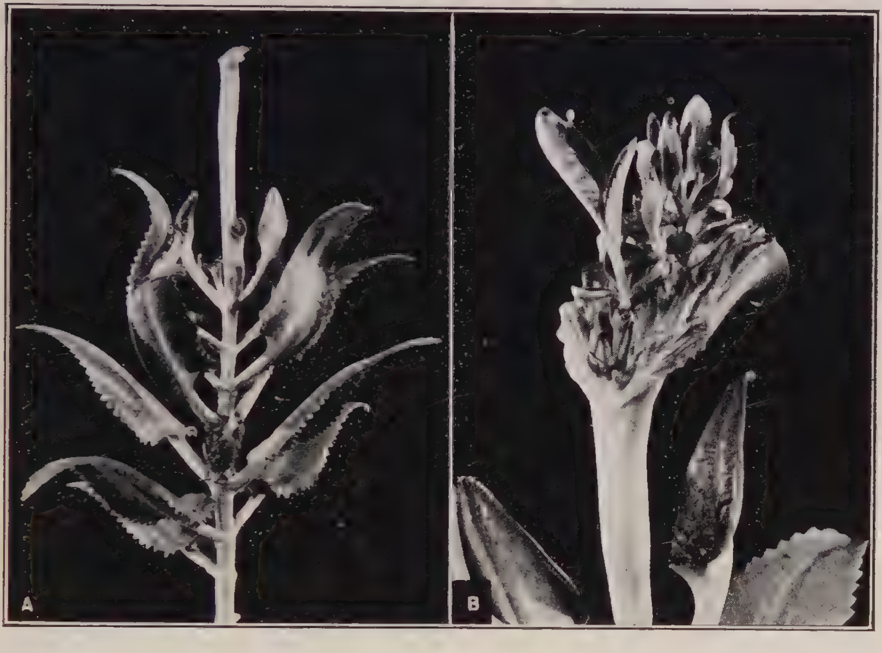

FIGURE 2. *Kalanchoe* plants modified with 4-chlorophenoxyacetic acid. A. Treated with 4-chlorophenoxyacetic acid, 5 mg. per g. of lanolin, and photographed after 77 days. Note proliferation from which adventitious buds arose, smooth edge, tubular leaves, and abnormal correlation of organs. B. Same treatment as A but photographed after 66 days. Note plantlets arising and growing from ridges on fasciated leaf.

Natural correlation of all organs of the treated plants was modified. In addition to plantlets being released from dominance, axillary shoots became displaced sometimes arising near the middle of the internode. Both responses are illustrated in Figure 3. Frequently a special wing-like struc-

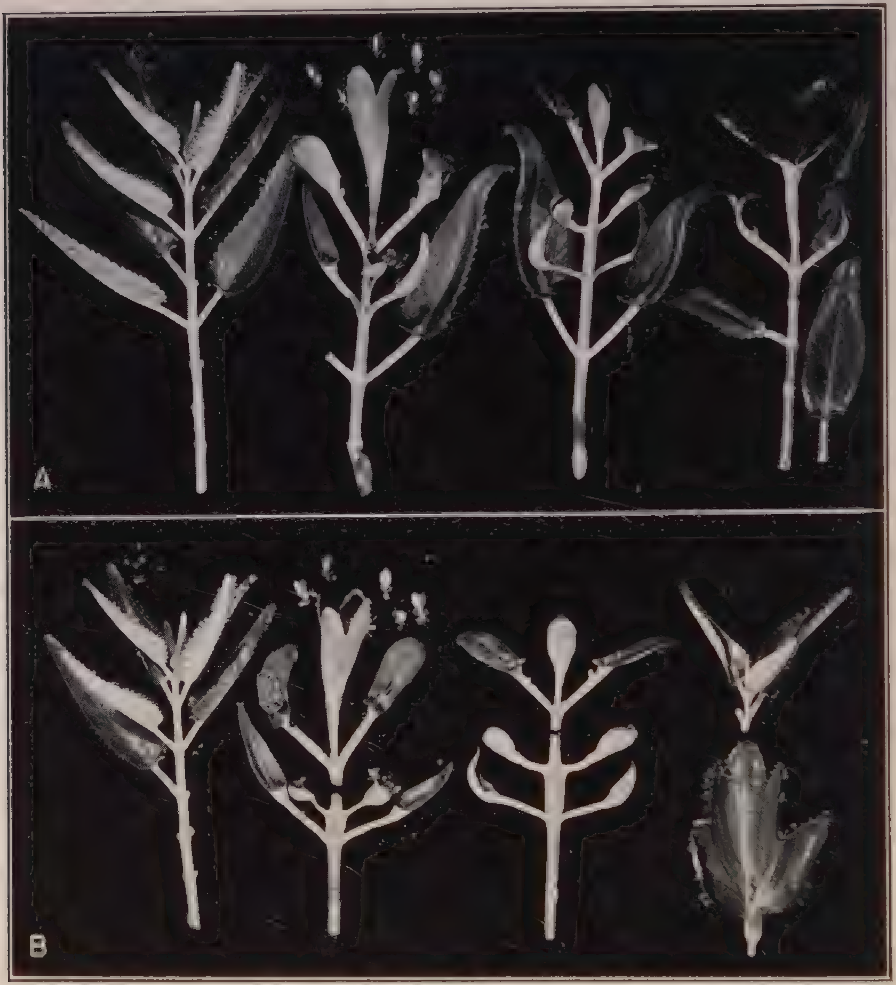

FIGURE 3. *Kalanchoe* branches showing change in correlation of organs and formative effects induced with 4-chlorophenoxyacetic acid at 5 mg. per g. of lanolin. The treatment was made in June and results photographed after 43 days. A and B. Controls on left, all others treated. Note displaced axillary shoots, funnel-shaped tops, change in correlation of plantlets, and ridges on face of leaves. Change in location of axillary growth is especially noticeable in B.

ture extended from the displaced shoot down toward the axil of the leaf. Plantlets appeared on the uppermost part of cup-like structures. These had no apparent resting period but continued development from the first. The comparative size of plantlets at the same age is shown in control and treated plants in Figure 3. The treated plants shown in this illustration have the terminal bud embedded in the fasciated basal part of the leaves.

This may account for the lack of apical dominance of the plantlets. However, similar effects show within two weeks after plants are treated and before the terminal bud becomes displaced. The releasal of the plantlets, therefore, may be directly related to the influence of the chemical applied. This influence could be direct or indirect since the immediate effect of the chemical might be inhibition of the terminal bud.

Upon recovery axillary growth usually appears first on the older portion of the plant. Frequently the first few leaves show some modification, but normal leaves appear later. This response is illustrated in Figure 3 B.

Adventitious buds grew on proliferations resulting from treatment with 4-chlorophenoxyacetic acid. The proliferation and the beginning of adventitious buds are illustrated in Figure 2 A. After the picture was taken a large number of adventitious buds appeared at various places on the proliferated tissue. This is in contrast to proliferated tissue resulting from treatment with 3-chlorophenoxyacetic acid, in which case adventitious roots appeared. High concentrations of 2-chlorophenoxyacetic acid caused slight proliferation but no adventitious buds appeared. These three isomers have the same empirical formula but cause different responses when applied to *Kalanchoe* plants.

2,4,5-Trichlorophenoxyacetic acid had a tendency to induce proliferations and adventitious roots but no modification of the leaves, whereas 2,4,6-trichlorophenoxyacetic acid did not induce proliferations or roots but did modify the top of the plant. The propionic and butyric acid homologs of 2,4,6-trichlorophenoxyacetic acid also modified leaves, induced cone-shaped cups, and caused other monstrosities on *Kalanchoe* similar to those caused by 4-chlorophenoxyacetic acid.

Xylenoxy compounds caused neither modification of leaves nor cup formation in *Kalanchoe* plants. The following compounds were used at 0.5, 1.0, and 2.0 per cent in lanolin:

3,5-Dimethylphenoxyacetic acid
α-(3,5-Dimethylphenoxy)propionic acid
α-(3,5-Dimethylphenoxy)butyric acid
2,5-Dimethylphenoxyacetic acid
α-(2,5-Dimethylphenoxy)propionic acid
α-(2,5-Dimethylphenoxy)butyric acid
2,4-Dimethylphenoxyacetic acid
α-(2,4-Dimethylphenoxy)propionic acid
α-(2,4-Dimethylphenoxy)butyric acid
3,4-Dimethylphenoxyacetic acid
α-(3,4-Dimethylphenoxy)propionic acid
α-(3,4-Dimethylphenoxy)butyric acid

Phenoxyacetic acid was not effective, but the propionic and butyric homologs caused modification of leaves and cone-shaped cups similar to 4-chlorophenoxyacetic acid.

Two derivatives of benzoic acid, 2,3,5-triiodobenzoic acid and 2-chloro-3,5-diiodobenzoic acid, both cause leaf modification and cone-shaped cups on *Kalanchoe* plants. Only a few of the substituted benzoic acids have been tested. Probably many others will be found effective. It is interesting to note that two different groups of hormone-like substances—substituted phenoxy and benzoic acids—bring about similar effects when applied to plants.

Indolebutyric acid is a well-known root-inducing substance but has no formative influence. When applied around the stem of *Kalanchoe* plants adventitious roots appeared within 12 to 14 days. When indolebutyric acid and 4-chlorophenoxyacetic acid were mixed and applied to the young part of the stem of *Kalanchoe* plants, both adventitious roots and formative effects resulted. Therefore, each chemical has its specific effect even though two (or perhaps more) are applied at the same time.

DISCUSSION AND SUMMARY

Some but not all of the substituted phenoxy acids have formative effects when applied to *Kalanchoe* plants. Two striking differences appeared when 4-chlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid are compared. The former caused a change in correlation of organs, hyponasty of leaves, modification of leaf pattern, and pronounced monstrosities at the terminal part of the plant. In contrast, the latter caused epinasty of leaves and root formation of treated stem tissue but no monstrosities. Similar differences appeared when these two chemicals were tested on Turkish tobacco (*Nicotiana tabacum* L.) plants. When applied to tomato (*Lycopersicon esculentum* Mill.) plants, however, the effects are nearly indistinguishable (6). Such cases as these herewith cited make it appear that the molecular configuration of the substance must in some way interlock with the mechanism of the species to bring about a given response. The genetic constitution of the species—perhaps the gene—appears to be the determining factor. This is mentioned because tobacco and tomato are closely-related species, and yet they respond differently when tested with these substances.

One of the most common of responses to hormone-like substances is epinasty of leaves. Some of the substituted phenoxy and benzoic acids, however, cause hyponasty of *Kalanchoe* leaves, and others are ineffective for either response. This variation is illustrated in Figure 1. The most effective root-inducing substances caused epinasty but not modification of leaves. 2,4,6-Trichlorophenoxyacetic and 2,3,5-triiodobenzoic acids were ineffective for rooting but caused leaf modification. Sixteen dimethyl substituted phenoxy acids (xylenoxy acids) were ineffective for rooting or modification of leaves.

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EFFECTS OF SOME ORGANIC ACIDS ON THE GROWTH OF WHEAT ROOTS IN SOLUTIONS

Edward A. Prill, Lela V. Barton, and Marie L. Solt

There have been many investigations on the effects of natural and synthetic substances on the germination of seeds and growth of seedlings. Evenari (6) recently has reviewed the subject. An account of war-time German work is also available (11). The present paper is on the effects of certain organic acids (neutralized) and a few related compounds on the growth of young wheat seedlings. Many of these acids have been tested on seeds and seedlings in one way or another by other investigators. In the present work it was desired to obtain comparative results of the effects of the various compounds by the procedure previously used in this laboratory.

PROCEDURE

A detailed description of the procedure has been given in a previous publication (3). In brief it was as follows. Beakers containing the solutions to be tested were covered with perforated, paraffined cheesecloth. Wheat[1] (*Triticum* sp.) seeds were germinated on moist filter paper in the laboratory. When the roots measured 6 to 7 mm., the seedlings were transferred to the beakers containing the test solutions in such a way that the primary roots extended through the cheesecloth perforations into the solutions, while the attached seeds remained on top of the cheesecloth. After 64 to 68 hours' growth in the dark at a constant temperature of 20° C. the primary root length of each seedling was measured. In each case, the growth was calculated as per cent of the growth given by the control, the average initial length being subtracted from the final length in making the calculations. Duplicate lots of 25 seedlings each were used for each solution.

The basal solution (control) contained calcium nitrate, magnesium sulfate, and potassium dihydrogen phosphate in the concentrations of 0.02 molar of each. Each test solution contained in addition to the above concentrations of these three nutrient salts the concentration, expressed as millimolar (m*M.*), of an organic acid (neutralized with KOH) or other compound as indicated in Tables I, II, and III or in the text.

Most of the chemicals tested were obtained as pure compounds from chemical dealers. The others which were of practical grade or were synthesized at this Institute were purified until they had the melting points reported for the pure compounds.

The acids, with the exception of anthranilic, were neutralized with

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[1] Marquis, 1946 crop, from the North Dakota Agricultural Experiment Station, Fargo, N. D.

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KOH solution prior to mixing with the components of the basal solution. The polycarboxylic acids were adjusted to pH 4.3 which was about the same pH as that of the basal solution.

RESULTS

The results obtained with the compounds studied over a range of concentrations are given in Table I. Both of the percentage values obtained with the duplicate beakers are recorded.

TABLE I

GROWTH OF WHEAT ROOTS IN NUTRIENT SOLUTIONS WITH DIFFERENT ORGANIC ACIDS OR RELATED COMPOUNDS

Acid*	Growth, as % of control, at various mM. concns.											
	0.002		0.01		0.05		0.25		1.25		6.25	
(Coumarin)	91	91	53	54	21	19	3	4				
Melilotic	86	92	41	34	10	8	2	2				
trans-Cinnamic	91	95	96	94	20	17						
Phenylpropiolic	93	97	57	53	13	13						
Hydrocinnamic	82	80	58	57	3	3						
(Hydrocinnamide)	96	94	89	91	88	88	87	84				
Piperic	111	113	65	71	1	0						
Tetrahydropiperic	33	40	11	10	1	1						
Benzoic	106	105	89	91	14	22						
Salicylic	82	80	46	49	15	17						
Anthranilic	92	85	95	95	42	38						
2-Furanacrylic	86	86	30	23	1	1						
Furoic	109	107	101	111	100	106						
Formic							46	49	2	1	0	0
Acetic							108	104	20	14	0	0
Propionic			108	108	44	51	6	6				
Butyric			84	95	76	79	4	3				
Caproic	89	105	99	102	65	72	1	1				
Capric			85	89	0	0	0	0				
Crotonic	107	106	112	108	84	82	11	12	1	1		
Malonic							114	117	108	113	41	43
Succinic							103	97	80	77	68	75
Adipic					94	94	73	73	45	45	12	12
Fumaric							86	86	81	77	68	73
l-Malic							81	80	58	63	58	60
Citric							83	78	78	73	49	49
d-Tartaric									74	81	23	31
Ethylenediamine-*N*, *N*, *N′*, *N′*-tetraacetic**	86	84	72	72	88	89	74	78				

* The acids were neutralized as described in the text.

** Obtained under the trade name, "Sequestrene" AA, from Alrose Chemical Co.

The results obtained with some other compounds tested only in a preliminary manner are as follows. Phorone, isophorone, and furfurylideneacetone at 0.1 mM. concentration and curcumin at 0.05 mM. concentration caused no inhibitions, while anisylideneacetone at 0.1 mM. concentration caused a slight inhibition (68 per cent of the growth of the control).

The above compounds are unsaturated ketones. 2-Hydroxyquinoline, the tautomeric form of which (2-quinolone) may be considered isosteric with coumarin, gave no inhibition at 0.2 m*M.* concentration. Maleic acid gave no appreciable inhibition at 2.0 m*M.* concentration.

TABLE II

ATTEMPTS TO OVERCOME INHIBITIONS WITH *l*-MALIC ACID*

Inhibitor, m*M.*	Growth, as % of control, with various m*M.* concns. of *l*-malic acid added							
	None		0.05		0.25		1.25	
None	100	100	90	93	74	75		
					56	57	55	49
Coumarin 0.0125	40	45			35	34	34	33
Coumarin 0.01	60	59	53	55	46	47		
Coumarin 0.05	23	23	19	20	17	16		
Melilotic acid 0.01	58	58			51	51	39	40
Piperic acid 0.01	66	61			45	48	37	37

* See footnote *, Table I.

Table II gives the results of experiments designed to determine whether or not *l*-malic acid can overcome the effects of certain inhibitory compounds. The results are clearly negative. Table III gives similar negative results of experiments in which thiourea was used in place of the *l*-malic acid.

TABLE III

ATTEMPTS TO OVERCOME INHIBITIONS WITH THIOUREA*

Inhibitor, m*M.*	Growth, as % of control, with various m*M.* concns. of thiourea added									
	None		0.05		0.25		1.25		5.0	
None	100	100	91	94	97	94	97	96		
							94	98	79	78
Coumarin 0.0125	40	45					21	26	17	18
Coumarin 0.01	60	59	48	42	38	44	33	34		
Coumarin 0.05	23	23	14	14	8	10	7	7		
Melilotic acid 0.01	58	58					52	54	42	44
Piperic acid 0.01	66	61					48	50	23	22

* See footnote*, Table I.

In all these tests the growths of the coleoptiles (not recorded in the tables) were not much different from the controls except when the root growths were reduced to very low percentages, in which cases the coleoptile growths also were reduced somewhat but not to correspondingly large degrees.

DISCUSSION

Coumarin (the lactone of *o*-hydroxy-*cis*-cinnamic acid) and certain other unsaturated lactones have become of considerable interest as inhibitors (1, 2, 17) of germination and growth. Also in the present study (Table I) coumarin was found to be strongly inhibitory. Marx *et al.* (10) reported that 3,3′-methylene-bis(4-hydroxycoumarin) was even more inhibitory than coumarin. Geiger and Conn (7) tested certain unsaturated ketones for antibiotic activity and considered them to act through the same mechanism as certain antibiotic unsaturated lactones. The results of the preliminary tests on several unsaturated ketones, mentioned above in this paper, show that these particular compounds are not strongly inhibitory to the growth of wheat roots. Certain quinones (8), particularly α- and β- naphthaquinones, have been reported to be very inhibitory to the growth of cress seedlings.

It is seen from Table I that most of the organic acids which contain an aromatic nucleus exert inhibitory effects of the same order as that of coumarin. Melilotic acid (*o*-hydroxyhydrocinnamic acid) is chemically related to coumarin and is at least fully as inhibitory as the latter. The related acids, *cis*-cinnamic, phenylpropiolic, and hydrocinnamic, are all strongly inhibitory but *cis*-cinnamic is the least of the three. A comparison of piperic and tetrahydropiperic acids again shows the acid having the saturated side chain as being the more inhibitory. Hydrocinnamide shows very little inhibitory effect although the acid from which it is derived is strongly inhibitory. Of the acids containing a furan nucleus, 2-furanacrylic acid is strongly inhibitory but furoic acid is not.

In 1944 Schmid (13) called attention to the fact that the inhibitors of germination and growth were not limited to unsaturated lactones and similar unsaturated compounds. It has been postulated that the unsaturated lactones (5, 7, 17) and also the unsaturated ketones (5) cause inhibitions by virtue of the addition reaction of an essential sulfhydryl group of an enzyme with the activated ethylenic double bond of the unsaturated compound. In the case of those highly inhibitory acids which do not contain an ethylenic double bond, such as, for example, melilotic, hydrocinnamic, and tetrahydropiperic, such a reaction obviously is not possible; therefore, at least for such acids, some other mechanism must be involved in causing inhibition.

Traube and Rosenstein (18) suggested that the inhibitory effects of compounds may be related to their ability to lower the surface tension of water. In a homologous series of compounds, according to Traube's rule, the ability to lower the surface tension is tripled by each additional CH_2 group (15, p. 1630–1632). Of the fatty acids which are next listed in Table I, formic, perhaps due to its somewhat aldehydic properties, is somewhat more inhibitory than acetic; however, propionic, butyric, caproic, and capric are more inhibitory than acetic and somewhat as would be expected on the basis of the above hypothesis. In fact, the inhibitory activity of capric acid appears to be of the same order as that of the above highly inhibitory acids which contain aromatic nuclei and which, presumably, are also quite surface-active.

In a previous publication (12) it was shown that some surface-active agents, such as detergents, in which the various attributes of surface activity are extremely pronounced, are also very inhibitory to the growth of wheat roots.

Crotonic acid has been reported to be naturally present in certain soils and to inhibit the growth of plants (14). In the present tests it was found to inhibit to about the same degree as butyric acid.

The polycarboxylic acids which are listed last in Table I show relatively little inhibition. Each of these would be expected to have relatively little surface activity in view of the presence of several hydrophylic polar groups and only a small lipophylic hydrocarbon portion in its molecular structure. Thimann and Bonner (16) reported that certain di- and tri-carboxylic acids (neutralized), such as citric, isocitric, succinic, fumaric, and malic, which are recognized as taking part in the Krebs cycle, and also malonic and maleic, which are foreign and even blocking to this cycle, all promote the growth of isolated *Avena* coleoptile sections and reverse the inhibitory action of iodoacetate on these. Bonner (4), however, found that malonic acid interferes with the respiration of the *Avena* coleoptile. He concluded that the results of his various experiments were in accord with the hypothesis that respiration in the *Avena* coleoptile proceeds through a system generally similar to the Krebs cycle.

The attempt to overcome the inhibitory effects of coumarin and other inhibitors on the growth of wheat roots with *l*-malic acid (Table II) was unsuccessful. Similarly, the failure to overcome these inhibitory effects with thiourea (Table III) is in contrast to the success reported by Lavollay and Laborey (9) in decreasing the inhibitory effect of coumarin on the germination of barley with thiourea. Perhaps similar results should not be anticipated for different situations. Recently Thimann and Bonner (17) reported that the inhibitory effects of coumarin and protoanemonin on the growth of plants can be partially overcome by dimercaptopropanol (BAL).

Evenari (6, p. 175–176) states that germination- and growth-inhibition are nearly always associated with one another; however, he cites references to several exceptions and concludes that the data are too scant to decide the question as to the identity of the two phenomena. Audus and Quastel (2), however, found that coumarin is usually more effective than 2,4-dichlorophenoxyacetic acid for the inhibition of germination but that the reverse is true for the inhibition of root growth. Thimann and Bonner

(17, p. 272) state, "However, seed germination is an obscure process and appears to involve root elongation, which is auxin-sensitive, as well as the complex of enzyme activations participating in germination proper." Audus (1, p. 217) states, "It is becoming more and more obvious, as new facts concerning the responses of plants to growth substances are brought to light, that their action in the complex phenomena of growth is a multiple one."

SUMMARY

A number of organic acids (neutralized) of various types and a few related compounds have been tested at a series of low concentrations (expressed in terms of millimolarity) for their effects on the growth of wheat roots in a solution of nutrient salts.

The inhibitory effects of the following aromatic acids were found to at least equal that of coumarin: melilotic, phenylpropiolic, hydrocinnamic, piperic, tetrahydropiperic, salicylic, and 2-furanacrylic. The somewhat less inhibitory aromatic acids were *trans*-cinnamic, benzoic, and anthranilic. Furoic acid was not found to be highly inhibitory. Hydrogenation of the double bonds in unsaturated acids, such as *trans*-cinnamic and piperic, appears to increase the inhibitory effects; in fact, tetrahydropiperic acid was the most inhibitory of all the compounds tested. Hydrocinnamide, in contrast to hydrocinnamic acid, is not highly inhibitory.

Results of preliminary tests on a few unsaturated ketones and 2-hydroxyquinoline suggest that these are not generally highly inhibitory.

In the fatty acid series, formic, propionic, butyric, caproic, and capric were found to be appreciably more inhibitory than acetic which was only slightly inhibitory. Crotonic acid appeared to be about as inhibitory as butyric acid.

Succinic, fumaric, *l*-malic, and citric acids, which are known to take part in the Krebs cycle, and also other polycarboxylic acids comprising maleic, malonic, adipic, *d*-tartaric and ethylenediamine-*N*,*N*,*N′*,*N′*-tetraacetic acids were found to show relatively little inhibitory effects.

The inhibitory effects of coumarin, melilotic acid, and piperic acid could not be reversed by *l*-malic acid or thiourea.

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RAPID FORMATION OF HIGH CONCENTRATIONS OF HYDROGEN SULFIDE BY SULFATE-REDUCING BACTERIA[1]

Lawrence P. Miller

Sulfate-reducing bacteria (*Desulfovibrio desulfuricans* [Beij.] K. and v.N.) have been investigated extensively since the early work of Zelinski (21) and the Dutch scientists Beyerinck (3) and Delden (7). This organism is very widely distributed and is of considerable economic importance in view of its possible connection with the genesis of petroleum deposits (22, p. 160), the large sulfur deposits in the salt domes of Texas and Louisiana (22, p. 112), and perhaps even some deposits of metallic sulfides (17, p. 11). The sulfate reducers are also of importance because of the problems arising from the unpleasant odor of H_2S, its high toxicity to most other forms of life, and especially its corrosive nature. Over 200 references have been reviewed by Starkey and Wight (17), with special emphasis on the role of these bacteria in corrosion. Kühr *et al.* (11) consider sulfate reduction to be one of the most commonly occurring and extensive microbiological processes on the earth.

As a result of the activity of the sulfate reducers in connection with the corrosion of submarine cables shipped under water the British Non-Ferrous Metals Research Association sponsored investigations which led to the discovery of a flavine dye which inhibits bacteria producing hydrogen sulfide under service conditions (14). This dye has also been useful in controlling the bacteria in the water used for washing wheat in a flour mill (2).

Some investigators have considered the halophilic, mesophilic, and thermophilic forms each to represent different species but the work of Baars (1) tended to show that they were strains of the same species. Baars also made important studies on the nutrition of these organisms and showed, for example, that the utilization of lactic acid to reduce sulfate may be represented by the following equations:

$$CH_3CHOHCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 4H$$
$$\tfrac{1}{2}H_2SO_4 + 4H \rightarrow \tfrac{1}{2}H_2S + 2H_2O$$

A species studied by Baars was able to oxidize acetate to CO_2 and H_2O and thus produce one and one-half moles of hydrogen sulfide, instead of

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one-half mole for each mole of lactic acid used. These relationships were established by Baars by careful quantitative studies of the amount of lactate used and the amounts of acetic acid, CO_2 and H_2S formed.

Since hydrogen sulfide is highly toxic to most forms of life the maximum concentration that can be produced by sulfate reducers is of special interest and has received considerable study. The maximum values that have been reported are those obtained by Baars (1). Working with nutrient media containing sufficient sulfate to yield over 2000 mg. of H_2S per liter, the maximum concentrations reached were 1448 mg. per liter for the halophilic forms, 794 for the mesophilic forms, and 566 for thermophilic strains. Rittenberg (13) concluded that concentrations of 1000 mg. per liter or less were limiting for his marine forms.

ZoBell (22) states that Daniltchenko and Tschigirine (6) found that at the bottom of the Black Sea sulfate reduction was so intense that the concentration of H_2S reached 6000 ml. per liter calculated at 760 mm. pressure and 0° C. Examination of the original report (6) reveals that there has apparently been some confusion because of the use of a comma rather than a period to indicate the decimal point and that these authors actually found a maximum of only a little over 6 ml. per liter. Vigorous sulfate reducers obtained by Issatchenko (9) from the Black Sea produced 300 to 500 mg. per liter of H_2S in laboratory cultures.

Of interest also is the rate of H_2S production. The growth of most species of bacteria under good laboratory conditions is usually rapid. In contrast, newly inoculated cultures of sulfate reducers may show a lag period of a number of days and the hydrogen sulfide produced may not reach its maximum for as long as two or three weeks (13). Van Niel (18), on the basis of his observation that NaCl increased the tolerance of purple sulfur bacteria to sodium sulfide, and of Baars' (1) report that higher salt concentrations and lower temperatures increased the H_2S production of the sulfate-reducing bacteria, suggested that a decrease in activity of the catalytic system goes hand in hand with a desensitization against hydrogen sulfide. This point was investigated by Rittenberg (13) who considered that he obtained confirmation of Van Niel's hypothesis although his data include a notable exception, namely, a culture which had an abnormally high rate of H_2S production, and yet produced a larger total amount of H_2S at a lower salt concentration than other cultures in the same series containing more salt. There is evidence that with other species of bacteria, certain conditions, such as temperature, for example, which are optimum for growth in terms of rapidity of cell division are not always the same as those optimum for the attainment of maximum numbers of cells per unit volume (10, p. 57).

Sulfate-reducing bacteria develop readily with inorganic nitrogen as a nitrogen source but it has been rather difficult to obtain growth on solid media from enrichment cultures until the work of Starkey (15) who used a medium containing 5.0 g. peptone and 10.0 g. dextrose per liter in addition to sulfate, agar, and a trace of $FeSO_4$. Rittenberg (13) found that a peptone-lactate medium functioned just as well as the peptone-dextrose medium for obtaining surface colonies, thus indicating that the dextrose was not essential but that peptone favors the growth of these bacteria under the conditions of these tests. Some development of the sulfate reducers will take place in media containing peptone and no added sulfate (1, 15) but Rittenberg (13) believes this to be due to sulfate present as an impurity in commercial peptones. No studies seem to have been carried out on the effect of peptone on the *rate* of growth of the sulfate-reducing bacteria when added to liquid media.

Bunker (4) found that yeast, yeast water, sterile filtered yeast culture in wort, and wort itself stimulated growth of the sulfate reducers but also reports that when these tests were repeated several years later on the same strain, consistent evidence of such stimulation was not obtained. More recently Butlin, Adams, and Thomas (5), working in the same laboratory, reported that the addition of 0.1 per cent Difco yeast extract greatly stimulated growth. They found that their medium including the yeast extract gave "consistently rapid and abundant growth, considerable turbidity being obtained within 48 hrs. with both mesophilic and thermophilic cultures." No numerical data are given.

While it is not ordinarily possible to obtain pure cultures of bacteria by enrichment techniques alone this possibility is of especial interest in the case of sulfate-reducing bacteria since H_2S, the product of their metabolism, is harmful or lethal to most forms of life. Rank (12) sometimes obtained pure cultures by allowing enrichment cultures to stand four or five weeks. Starkey (15) worked with cultures kept at 30° C. for 443 days without being transferred and found that they were free of any contamination after this period, although there was no certainty that the cultures were pure when originally prepared. Rittenberg (13) was unable to obtain pure cultures by enrichment techniques alone under conditions in which he obtained a maximum of about 900 mg. per liter of H_2S.

In the experiments reported in the present paper emphasis has been placed on devising nutrient media giving high concentrations of H_2S. About 40 strains of bacteria have been used, including some obtained from previous workers, some from core material and drilling mud from the operations of the Texas Gulf Sulphur Company, and some from the ocean at Newport, R. I., and soils from a variety of locations. Nutrient media designed to make possible high yields of H_2S must necessarily contain a considerable concentration of salts. There has been no difficulty in getting strains from non-marine environments to develop in relatively high concentrations of sodium chloride or other salts. No evidence was obtained that the nutrient requirements for the rapid production of high concentrations of H_2S differed for strains originating from dissimilar environments. By using media containing ample quantities of sulfate and lactate, and $CaCO_3$, concentrations of H_2S considerably over 2000 mg. per liter were readily obtained. With such media concentrations of 1200 to 1400 milligrams of H_2S per liter were often obtained with crude cultures after only one or two transfers even though the cultures included contaminants which produced much turbidity when inoculated into a nutrient medium maintained under aerobic conditions. After further transfers to make a total of six or seven, development in the aerobic nutrient broth usually no longer took place, thus indicating the absence of facultative organisms which have been reported to be the most difficult to eliminate from cultures of the sulfate reducers (13).

Absence of contaminants developing in a medium maintained under aerobic conditions did not necessarily result in increased H_2S production much above 1200 to 1400 mg. per liter. In studying the factors associated with the production of a high concentration of H_2S, it became apparent that, contrary to what is indicated by some of the literature cited above, high total production was associated with rapid rates of H_2S formation. Thus if cultures were transferred into appropriate media every three or four days instead of every 10 to 21 days or longer, vigorous cultures producing large amounts of H_2S readily resulted. *All* the strains included in these studies were carried through such a procedure and were then able to produce over 2000 mg. per liter in three or four days.

Peptone, yeast extract, and other adjuvants have been found to be powerful stimulants for the growth of the sulfate reducers. The effect sometimes appears to be qualitative in that the presence of such additives seems to extend the temperature range, the pH range, and the concentration of salts tolerated. These results with stimulants and inhibitors and combinations of the two will be reported in subsequent papers.

MATERIALS AND METHODS

CULTURE TECHNIQUES

The bacteria were grown either in Pyrex bottles of about 55-cc. capacity with interchangeable glass stoppers or in 2-oz. flat prescription bottles, actual capacity 65 to 70 cc., with plastic caps. When the latter were used it was necessary to examine the caps after each use to eliminate cracked caps and to see whether the cap lining remained intact. When necessary a layer of tin foil was added to the cap lining to permit tight closure of the bottle. If closure was not sufficiently tight a deposit of sulfur would be found at the neck due to the oxidation of H_2S, and contamination with aerobic bacteria was encouraged. Sometimes enough pressure developed in a culture to crack the bottle. This happened infrequently and was not a deterrent to the use of this type of bottle. Since 400 to 600 cultures were usually being grown at one time during the course of these experiments the availability of the prescription bottles considerably decreased the cost of the culture flasks required.

With both types the bottles were sterilized after being filled with medium to about $\frac{5}{6}$ of their capacity and fitted with a cotton plug. The stoppers or caps and extra medium to fill the bottles were sterilized separately. After sterilization for 20 minutes at 15 lbs. of steam pressure the bottles were cooled rapidly in cold tap water. Other workers have usually stated that their media were cooled immediately before use (13, 17). In the present experiments freshly prepared media were used for the rest of the day without re-heating and cooling and no difference was noted between the results obtained with freshly cooled media and media that had stood for some hours. As a matter of fact preliminary tests indicate that media which have stood for several days will give about the same results as fresh media under the conditions of these experiments which usually involved inoculation with 1 cc. of a culture containing over 1000 milligrams of H_2S per liter into 55 to 70 cc. As a routine, only media prepared the same day were used. If media left over from a previous day were to be used, they were re-sterilized. Usually utilization of such media was avoided since the pH was lowered with each sterilization.

To start the cultures, 1 cc. of a previous culture, or less in some cases in which a long series of comparative cultures were set up, was added to each bottle with aseptic precautions, after which a flamed crystal or crystals of ferrous ammonium sulfate were dropped into the culture, the bottle completely filled with the medium, and the stopper or cap put on. As a routine procedure during most of this work each time transfers were made from a culture to set up the usual anaerobic cultures, 1 cc. of inoculum was also transferred to a special medium, described below, which was maintained under aerobic conditions. This latter culture served as a means of estimating the degree of purification achieved by repeated transfers from media in which high concentrations of H_2S had been reached. When growth no longer took place in this medium under aerobic conditions, absence of contaminating facultative organisms was indicated. Even after a given strain seemed to be free of such contaminants a culture in this medium was started each time transfers were made in order to have a check on the sustained freedom of the strain from contaminants. All cultures were kept in a room or oven thermostatically controlled at 30° C.

DETERMINATION OF THE HYDROGEN SULFIDE PRODUCED

For the determination of the amount of H_2S produced by the cultures a measured quantity, usually 50 cc., was rapidly mixed with an excess of

N/10 iodine solution in the presence of acetic acid and the excess iodine determined by titration with N/10 sodium thiosulfate with starch as an indicator. It was not practical to use a pipette to measure out the portion to be titrated since the suction necessary caused a loss of H_2S and therefore a graduated cylinder was used. It is important that the manipulations up to the time an excess of iodine is present be carried out rapidly to decrease loss of H_2S by diffusion into the air. This is especially the case when amounts of H_2S above 1700 mg. per liter are involved. As a matter of fact such cultures can immediately be recognized on opening and on pouring the solution into a cylinder since bubbles of gas are seen to escape. To reduce losses of H_2S, cultures expected to be high in H_2S content were usually cooled for several hours or even overnight in a room maintained at about $-5°$ C. before being titrated. It was a routine procedure throughout all these experiments to determine quantitatively the amount of H_2S produced in all cultures in which any growth took place. In cases in which transfers were made the amount to be transferred was withdrawn as rapidly as possible after opening the culture vessel and a measured quantity of the remainder of the culture solution taken immediately for titration with iodine.

In the early experiments the amount of H_2S produced was usually determined after the cultures had been maintained for 10 days to 3 weeks or more. The cultures were observed frequently to note evidence of blackening resulting from the formation of ferrous sulfide from the ferrous ammonium sulfate added and for turbidity caused by the growth of the bacteria. Thus an indication of the time necessary for active growth to start was obtained as well as a figure for the total amount of H_2S present at titration time.

Frequently when media were tested which turned out to be somewhat unfavorable the lag period was increased but the total amount of H_2S produced was not necessarily less than that reached in a more favorable medium. It thus became apparent that when comparisons between several media were to be made it was desirable to make determinations of the H_2S content at several time intervals since often a given medium had an effect on the rate of H_2S production which was obscured if determinations of H_2S content were not made until all cultures in the series had attained their maximum content. If three or more properly spaced points are available it is possible to plot the H_2S production against time and thus determine the time necessary to reach 50 per cent of the total. This 50 per cent point can serve as a convenient means of comparing several media. It is of course necessary to use a separate culture for every point when such rate studies are made.

In some cases in which it was known or suspected that the media contained substances other than H_2S which react with iodine under the conditions employed for the determination of H_2S, a stream of nitrogen was passed through the media after acidification with acetic acid or HCl and then through Van Slyke-Cullen tubes (19) containing a 2 per cent solution of zinc acetate. After it was apparent that all of the H_2S had been driven off into the zinc acetate solutions the H_2S was released from the ZnS by the addition of HCl and then titrated with iodine and sodium thiosulfate in the usual way. It is essential to allow sufficient time for the HCl to dissolve the ZnS which may take some minutes.

Since in routine transfers culture vessels of 50 to 70 cc. capacity were inoculated with 1 cc. of a culture containing up to 2500 mg. of H_2S per liter it is apparent that values below 50 mg. per liter may be the result of the H_2S present in the inoculum rather than bacterial development. Low values are frequently included in the tables presented in order to show lack of development in a given culture as compared to a culture in a more favorable medium, or, in time series, to show more clearly when growth started. In the case of original inoculations low values for H_2S are again of little significance since small amounts of H_2S, or other substances reacting with iodine, may arise from sources other than the reduction of sulfate. With original inoculations results were considered positive only if growth occurred on transfer.

Values are reported in terms of milligrams of H_2S per liter. Included in this figure calculated as H_2S is the small amount of sulfide present as ferrous sulfide as the result of the trace of ferrous ammonium sulfate added to each culture.

SOURCE OF THE STRAINS OF BACTERIA

Non-marine strains of the bacteria were obtained from Prof. R. L. Starkey of Rutgers University who originally brought them from Holland. These have been carried through the experiments and are designated in the tabular material presented as S1, S2, and S3. Marine forms were furnished by Prof. Claude E. ZoBell of the Scripps Institution of Oceanography. These are designated Z2 and Z3. Numerous strains were obtained from core material and drilling mud from the workings of the Texas Gulf Sulphur Company in Texas and the numbers assigned to them are given in a later section in which the techniques used in obtaining cultures from these sources are described. Cultures were also obtained from stagnant fresh water, from the ocean at Newport, R. I., and from soil at the Institute and other locations. These will be specifically designated in later sections if such designation is indicated. As stated in the introduction, media containing sufficient sulfate to make the formation of high concentrations of H_2S possible necessarily contain a considerable concentration of salts. It was found that the non-marine strains will grow readily in such solutions even on the first transfer. Thus within the scope of the experiments reported herein, *i.e.* for the rapid production of high concentrations of H_2S, the same media serve equally well for both forms. Ability to form and withstand high concentrations of H_2S seems to be equal for the non-marine and marine forms in contrast to the results of Baars (1). Thus insofar as this particular point is concerned, the various lines carried along and referred to as strains apparently do not differ.

NUTRIENT MEDIA EMPLOYED

Since this report is concerned with the development of a medium for the rapid formation of high concentrations of H_2S, many variations in the composition of media have necessarily been tested. To make the formation of large amounts of H_2S possible, ample sulfate and lactate had to be provided. Distilled water was used in making up the media rather than tap water or sea water, in order to have a closer control of the composition of the media being used. As a starting point variations in the medium employed by Starkey (17) were tested. Starkey's medium has the following composition:

NH_4Cl	1.00 g.
$MgSO_4 \cdot 7H_2O$	2.00 g.
Na_2SO_4	0.44 g.
K_2HPO_4	0.50 g.
$CaCl_2 \cdot 2H_2O$	0.10 g.
Sodium lactate	3.50 g.
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Trace
Tap water	1000 cc.

This medium was not designed for the production of high concentrations of H_2S since, not taking into account any SO_4 that may be present in the tap water added, the maximum concentration of H_2S that can be reached is 383 mg. per liter (assuming that the medium was made up to a volume of 1000 cc. which was the procedure used rather than adding 1000 cc. of water to the salts). If sufficient sulfate were present this medium could yield 532 mg. per liter based on the lactate content and assuming oxidation of the lactate to acetate only.

Another medium which is based on that used by Starkey for the growth of surface colonies of the sulfate reducers was much used in the work reported herein. It was made up as follows:

NH_4Cl	1.00 g.
$MgSO_4 \cdot 7H_2O$	1.50 g.
Na_2SO_4	0.66 g.
K_2HPO_4	0.50 g.
$CaCl_2 \cdot 2H_2O$	0.50 g.
Peptone	5.00 g.
Dextrose	10.0 g.
Sodium lactate	3.50 g.
Distilled water to	1000 cc.

Since Starkey had found that a medium containing organic matter in addition to sodium lactate and agar favored the growth of the sulfate reducers in surface colonies it was thought desirable to use such a medium in attempting to obtain cultures from core samples. The data from these tests are presented in a different section of this report. This medium (referred to as No. 22) was also used extensively, under aerobic conditions, to reveal the presence of contaminants in the enrichment cultures worked with. Crude cultures contained bacteria which showed vigorous development in this medium. After growth through a number of transfers in media in which a concentration of 1000 or more mg. per liter of H_2S was reached, bacteria developing in this aerobic medium were no longer present.

Because of the large number of ingredients in media used for growth of the sulfate reducers, possible variations are extremely large and it is by no means certain that the most favorable medium found in the present experiments is the best one. The most important variation introduced, aside from furnishing ample quantities of sulfate and lactate, is the addition of $CaCO_3$ which brings about a higher initial pH than used by former workers who recommend 7.0 (17) or 7.5 (13). Details as to the various media tested will be presented in the appropriate sections following. The medium (No. 220) at present used for the routine cultivation of the sulfate reducers and the best or one of the best so far tested has the following composition:

NH_4Cl	1.00 g.
$MgSO_4 \cdot 7H_2O$	2.00 g.
Na_2SO_4	18.25 g.
K_2HPO_4	0.50 g.
$CaCl_2 \cdot 2H_2O$	0.10 g.
Sodium lactate	21.0 g.
NaCl	10.0 g.
$CaCO_3$	1.00 g.
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Trace
Distilled water to	1000 cc.

This medium contains sufficient lactate to give 3192 mg. per liter of H_2S and sulfate to yield 4655 mg. of H_2S per liter if sufficient organic matter were available. Vigorous cultures growing in this medium will develop a concentration of H_2S of over 2000 mg. per liter (up to about 2500 mg. have been obtained) in three to four days.

CULTURES FROM SULFUR-BEARING DOMES

Drill core samples from various depths, usually about 700 to 900 feet, and often containing free sulfur, were obtained from sulfur-bearing salt domes in Texas. These samples were taken at the drilling sites without any aseptic precautions and were received in canvas bags in rather large pieces, sometimes 6 to 7 inches long with a diameter of 4 inches. To test for sulfate reducers the samples were wrapped in sterile cheesecloth, broken with a hammer, and then pieces taken from inside the cores for inoculation into suitable media. In some cases small pieces were further covered with sterile cheesecloth and pounded in a sterile mortar and a gram or two of the powdered material added to each of several bottles of culture medium.

TABLE I

PRODUCTION OF H_2S FROM MIXED CULTURES OBTAINED FROM 20 DRILL CORES FROM SULFUR-BEARING SALT DOMES AND INCUBATED IN THREE DIFFERENT MEDIA FOR ABOUT THREE WEEKS*

Drill core number	H_2S produced in 3 media, mg. per l.			
	Medium 4, Av.	Medium 22, Av.	Medium 51, Av.	Totals for core samples**
13	nb	80	86	828
14	nb	12	16	252
15	nb	36	74	548
16	nb	12	b	60
17	3	26	88	580
18	nb	96	92	939
19	nb	94	49	717
20	nb	29	b	143
21	nb	112	134	1229
22	nb	89	56	725
23	9	87	53	745
24	5	91	49	722
25	nb	78	136	1067
26	nb	53	29	410
27	nb	49	55	521
28	nb	28	20	240
29	nb	68	46	560
30	nb	97	54	756
31	nb	58	49	533
32	nb	39	36	373

* Medium No. 4 is Starkey's medium with 3% NaCl added; Composition of medium 22 is given in earlier section; No. 51 is No. 22 plus 3% NaCl. Values in columns under media numbers are averages for 5 replicated cultures in each case; nb indicates no bloom, b, bloom but no blackening and therefore no formation of H_2S.

** Quantities of H_2S, in mg. per l., produced in each culture in which development occurred, totalled for all media for each core sample.

These tests were carried out before the work on the development of improved media had been completed. Nevertheless growth was readily obtained when media containing peptone and glucose in addition to sodium lactate were used. The results obtained with 20 core samples are summarized in Table I. Fifteen bottles were inoculated with material from each core sample, including five inoculations for each of three media, namely, No. 4, Starkey's medium in 3 per cent NaCl solution, No. 22 containing peptone and glucose, and No. 51 which has the same composition as No. 22 but with 3 per cent NaCl added. All media were adjusted to pH 7.0 before autoclaving. Under these conditions, operating at pH values later found considerably below optimum, much better results were obtained with solutions 22 and 51 than with 4. As a matter of fact, if medium 4 only had been used it would no doubt have been concluded that sulfate reducers were essentially lacking from this material. However, considerable development occurred in media 22 and 51. The quantities of H_2S produced, expressed as milligrams per liter, in each culture in which development occurred were totalled for each core sample and these values are shown in column 5 of Table I. It is seen that this total varied from 1229 for core sample No. 21 to only 60 for sample No. 16. Transfers were made from some cultures from each lot and a number of these have been carried through the media experiments reported in this paper under numbers corresponding to those in Table I.

After removal of the material for the inoculations reported in Table I the core specimens were stored in sterile glass stoppered bottles. Further tests carried out with two of these samples and with four others are reported in Table II. In these tests irregular pieces with a maximum diameter of 2 to 3 inches were dipped into hot paraffin (135° C.) for 5 seconds, then covered with sterile cheesecloth, broken into small pieces in a sterile mortar, and portions inoculated into the same three media used for the tests reported in Table I. No development took place in medium No. 4 but considerable growth occurred in media 22 and 51. Many of these cultures grew on transfer and those carried through the experiments are designated with the letter P preceding the core sample number.

TABLE II

SUMMARY OF RESULTS OF ORIGINAL INOCULATIONS INTO DIFFERENT MEDIA WITH DRILL CORE SAMPLES DIPPED FOR 5 SECONDS INTO HOT PARAFFIN (135° C.)

Medium No.*	Sample No. and mg. per l. of H_2S produced after various time intervals											
	3		6		7		11		22		24	
	Days	H_2S*	Days	H_2S	Days	H_2S	Days	H_2S	Days	H_2S	Days	H_2S
4	35	nb nb	35	nb nb	35	nb nb	36	nb nb	35	nb nb	35	nb nb
22	35	nb 21 55	35	61 26 41	36	94 77 75	36	131 94 153	35	nb 61 119	35	b 48 nb
51	35	34	35	78	36	55	36	104	35	nb	35	36
	35	38	13	58	13	41	36	92	13	29	35	41
	13	27	35	44	36	41	13	70	35	65	13	24
	21	51	21	84	20	49	36	107	21	51	35	31

* See footnotes to Table I for composition of media and for explanation of abbreviations.

In another experiment, pieces of core with a maximum diameter of 2 to 3 inches were immersed in paraffin maintained at 135° for 6 seconds, 60 seconds, and 10 minutes, respectively, and then inoculated into the three media. Subsequent development took place after 60 seconds of immersion but not at all or only to a slight degree after 10 minutes. The data are given in Table III. A strain obtained by transfer from No. 22 which had been subjected to 60 seconds' immersion has been made use of in the media work and has been designated PB22.

TABLE III

SUMMARY OF RESULTS OF ORIGINAL INOCULATIONS INTO DIFFERENT MEDIA WITH DRILL CORE SAMPLES IMMERSED IN HOT PARAFFIN (135° C.) FOR 6 SECOND TO 10 MINUTES

Medium No.*	Sample No. and mg. per l. of H_2S produced after various time intervals											
	8(6 sec.)		8(60 sec.)		8(10 min.)		22(6 sec.)		22(60 sec.)		22(10 min.)	
	Days	H_2S*	Days	H_2S	Days	H_2S	Days	H_2S	Days	H_2S	Days	H_2S
4	38	nb	38	nb	38	nb	38	nb	38	nb	38	nb
		nb		nb		nb		nb		nb		nb
		nb		nb		nb		nb		nb		nb
22	19	100	20	17	38	nb	38	32	38	56	38	nb
	19	54	19	37		nb		89		57		nb
	20	5	20	34		48		nb		107		nb
51	19	70	20	14	38	nb	38	nb	38	nb	38	nb
	19	38	20	14	38	nb	38	nb	38	nb		nb
	20	48	19	23	20	14	19	68	19	43		nb

* See footnotes to Table I for composition of media and for explanation of abbreviations.

Of interest also are the results obtained with samples of drilling mud used in the drilling operations. Two samples, one containing mud from a sulfur well and the other mud from the central mud pit, were received. One cc. portions were inoculated into each of three replicates of three different media as shown in the first part of Table IV. It is seen that both samples produced rather large amounts of H_2S in the first inoculation indicating the presence of large numbers of sulfate-reducing bacteria. A second test started 65 days later also gave good growth. Some of the cultures in media 4 and 11 reduced the greater part of the sulfate present in the media (the media contained sufficient sulfate to yield 383 mg. per liter of H_2S). Living bacteria were still present about 300 days after the receipt of the sample as indicated in the last part of Table IV. Although not so much development occurred as in the earlier inoculations, the bacteria grew readily on transfer.

It will be noted that in these tests the bacteria did not grow in medium 51 as shown in Table IV. Similarly in connection with the data obtained in the tests started 65 days later, no growth of sulfate reducers took place in media 22 and 51 (not shown in the table) while excellent growth took place in media 4 and 11. These results are in contrast to those found when core material was tested. The reason for this difference is not clear unless the nature of the contaminating bacteria is an important factor. Also the tests reported in Table IV were conducted at a somewhat higher pH in that the media were adjusted to give a pH of about 7.0 after autoclaving while in the earlier experiments the pH values were somewhat lower in that the pH was adjusted to 7.0 before autoclaving.

TABLE IV

DATA SHOWING THE PERSISTENCE OF SULFATE REDUCERS IN DRILLING MUD

Fresh Sample					After 65 days					After 295 days				
Medium No.*	At well		At central mud pit		Medium No.*	At well		At central mud pit		Medium No.*	At well		At central mud pit	
	Days	H_2S	Days	H_2S		Days	H_2S	Days	H_2S		Days	H_2S	Days	H_2S
21	30	222	30	14	4	35	325	33	312					
		138		218			344	33	351					
		222		293			298	33	145					
							348	19	329					
49	30	286	30	97	11	35	269	33	130					
		341	22	390			158	19	41	220	25	68	13	136
		409	30	319			97	33	135		41	34	41	nb
							80	33	137					
51	30	b	30	b	72	35	46	19	509					
		b		b			73	33	520					
		b		b			218	33	387					
							45	33	416					

* Composition of media used: 4, Starkey's medium plus 3% NaCl; 11, Starkey's medium; 21, Starkey's medium plus 24.3 g. Na_2SO_4, 10.5 g. sodium lactate, and 30 g. NaCl per liter; 49, NH_4Cl 1.0 g., $MgSO_4 \cdot 7H_2O$ 6.68 g., Na_2SO_4 1.47 g., K_2HPO_4 0.5 g., $CaCl_2 \cdot 2H_2O$ 0.20 g., sodium lactate 3.5 g., NaCl 30 g., $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ trace, water to 1000 cc.; 51, medium 22 (see section on media) in 3% NaCl; 72, same as 220 (see section on media) except 36.94 g. Na_2SO_4 instead of 18.25 g. plus 0.30 g. glucose and 0.30 g. peptone per l.

The results in the accompanying tables clearly show that sulfate reducers are associated in large numbers with the core samples from the sulfur-bearing salt domes. In view of the very rich possible source of bacteria from the drilling mud and their wide distribution in nature it would seem to be a mistake to place undue emphasis on any speculation as to the significance of the isolation of the bacteria from the inner portions of drill core material.

EFFECT OF SODIUM CHLORIDE

Previous workers have employed different media for marine forms and the non-marine forms. They have usually used sea water to make up the media for the former and tap water for the latter. In the present experiments the use of both tap water and sea water was avoided since it was desired to know more exactly the composition of the media being tested. However, it was necessary to give consideration to the possibility that the media requirements for the two forms might be different, and an obvious possibility is that the marine forms would be favored by the addition of NaCl which might on the other hand be unfavorable for the development of the non-marine forms. It is also necessary to consider the effect of high concentrations of other salts as well as NaCl since a medium must necessarily contain fairly high concentrations of sulfates (unless a sulfate of low solubility is present in the solid phase) for the production of high concentrations of H_2S. It is quite possible that the optimum concentration of NaCl might be different at different sulfate levels.

TABLE V

Growth of Two Non-Marine Strains on First Transfer to Media Containing Varying Amounts of Sodium Chloride

NaCl added to Starkey's medium, %	S2		S3	
	Time for growth to start, days*	H_2S as mg., l., after 18 days	Time for growth to start, days	H_2S as mg., l., after 31 days
0	4	290	2	303
1	4	293	3	304
2	4	305	7	294
3	5	303	4	332
4	8	249	6	303

* As shown by formation of ferrous sulfide from trace of ferrous ammonium sulfate present.

Considerable attention has been given by previous workers to the effect of NaCl on the growth of sulfate-reducing bacteria and some of this work is referred to in the introduction. Baars (1) was able to grow thermophilic forms under mesophilic conditions and the mesophilic forms at high temperatures. He was also able to grow fresh water forms in media containing 3 per cent salt and halophilic forms in the absence of salt. Usually these adaptations took place gradually. Rittenberg (13) worked with a halophilic strain which he was unable to acclimatize to media of low salt content or to temperatures above 40° C.

In the present tests it was found that non-marine forms obtained from Dr. R. L. Starkey and maintained for many years on media free of added NaCl developed readily at fairly high concentrations of salt on the first transfer. The data for two strains are given in Table V. It is apparent that both strains produced about the same amount of H_2S regardless of whether the medium contained salt up to 4 per cent. Growth was retarded several days by 4 per cent salt.

Transfers were made from some of the cultures in the S2 series and the effect of the salt content of the previous culture media on development in the subsequent media noted as shown in Table VI. The ready adaptation of these strains to salt is further confirmed by these data. Referring to the latter part of the table, it is seen that after one growth period in 4 per cent NaCl, production of H_2S took place more rapidly in 1 to 4 per cent NaCl than in Starkey's medium without NaCl. The total amounts of H_2S formed are about the same in the medium without salt as in the media containing from 1 to 4 per cent salt. It will be noted that considerable H_2S was produced in two cultures containing as much as 8 per cent NaCl.

The fact that these strains develop readily in the presence of considerable quantities of NaCl at the first transfer has also been confirmed in Starkey's laboratory (16).

TABLE VI

EFFECT OF THE NaCl CONTENT OF THE PRECEDING MEDIUM UPON THE DEVELOPMENT IN SUBSEQUENT CULTURE MEDIA OF VARYING NaCl CONTENT

NaCl added to Starkey's medium, %	Previous media and H_2S produced in present cultures					
	Starkey's		1% NaCl		4% NaCl	
	Time for growth to start, days	H_2S as mg./l., after 35 days	Time for growth to start, days	H_2S as mg./l., after 38 days	Time for growth to start, days	H_2S as mg./l., after 40 days
0	4	352	4	378	15	343
1	3	333	3	361	3	346
2	—	—	3	367	8	308
4	11	346	5	345	6	309
8	—	—	31	151	*	127

* Not observed.

These results indicate a lack of sensitivity to reasonable concentrations of NaCl by non-marine forms and suggest the possibility that the nutrient requirements of marine and non-marine forms may not be so different as was previously supposed. Accordingly all new media tested were used for both marine and non-marine forms and under the conditions of these experiments (working at rather high concentrations of total salts) no differences in requirements were apparent. The various media tested will be more fully discussed below but some further results of tests on the effects of NaCl will be given in this section.

The effect of the concentration of NaCl on the total amount of H_2S produced in a series of 11 cultures including 8 strains was tested using a medium (No. 104) which is similar to No. 220 except that it contains 36.5 g. of Na_2SO_4 instead of 18.25 g. and 1.70 g. of $CaCl_2 \cdot 2H_2O$ instead of 0.10 g. of the latter and contains no $CaCO_3$. This medium in addition to 2.1 per cent sodium lactate has a total salt concentration of 5.17 per cent.

TABLE VII

Effect of Varying the Concentration of NaCl in the Medium on the Quantity of H_2S Produced by Various Strains of Sulfate-Reducing Bacteria

Strain and transfer No.	NaCl content of medium transferred from, %	H_2S produced, mg. per liter			
		Days	Medium 125 (No NaCl)	Medium 104 (1% NaCl)	Medium 124 (3% NaCl)
C-13	1	11	622	1077	41
C-13A	0	16	797	895	358
S2-11	1	17	971	1104	997
S2-11A	0	18	724	1118	247
29-5	3	21	961	1186	775
24-6A	1	16	968	1055	659
24-5B	3	20	993	999	712
1M-4	1	16	1033	1457	1213
P11-5	1	17	1056	1309	1227
23-5	3	21	964	1043	879
R2-2B	3	21	1326	1344	1435
Totals			10415	12587	8543

Modifications tested were No. 125, containing no NaCl, and No. 124, with 3 per cent NaCl. The media were adjusted to pH 7.5 before autoclaving and had a pH of about 7 when inoculated.

The results are given in Table VII. Strains labelled C and S2 are non-marine in origin. It will be noted that the media used immediately preceding this test series varied as to NaCl content and included media free of salt as well as media containing 3 per cent NaCl. In every case but one the highest concentrations of H_2S were reached in the media containing 1 per cent NaCl. Examination of the data by the method of ranks (8, 20) shows the superiority of the medium with 1 per cent NaCl over the media containing either no added NaCl or 3 per cent NaCl to

TABLE VIII

Effect of Varying the Concentration of NaCl in the Medium on the Quantity of H_2S Produced by Various Strains of Sulfate-Reducing Bacteria ($CaCO_3$ Present)

Strain and transfer No.	H_2S produced, mg. per liter			
	Days	Medium 111 (No NaCl)	Medium 105 (1% NaCl)	Medium 110 (3% NaCl)
Z2-8A	12	1116	1562	1315
S2-8A	12	1056	925	21
Z3-9	13	1050	1230	55
C-12	18	992	1208	859
S2-10	17	673	1102	49
P11-4	19	1017	1605	1174
1M-3	19	751	1544	1595
24-5	18	1002	1186	334
Totals		7657	10362	5402

be highly significant. The latter two media do not differ significantly from each other.

The results of another experiment in which the effect of varying the quantity of NaCl was tested, are reported in Table VIII. Medium 105, containing 1 per cent NaCl, differs from No. 104, the composition of which is given above, in that it contains 0.10 g. $CaCl_2 \cdot 2H_2O$ per liter instead of 1.70 g. and includes in addition 1.12 g. $CaCO_3$ per liter. Media 110 and 111 differ from 105 only in their NaCl contents. In this case too the medium with 1 per cent NaCl is definitely superior to no NaCl or 3 per cent NaCl. In these tests the medium used immediately preceding this series was No. 21 which contains 3 per cent NaCl. Of the strains included S2 and C are non-marine in origin.

TABLE IX

EFFECT OF VARYING THE CONCENTRATION OF NACL ON THE RATE OF H_2S FORMATION BY SEVERAL STRAINS OF SULFATE-REDUCING BACTERIA

Concentration of NaCl, %*	pH	H_2S production								
		CS-15A			38-11			Z2-22		
		Time, hrs.	H_2S as mg./l.	Time to reach 600 mg., hrs.	Time, hrs.	H_2S as mg./l.	Time to reach 700 mg., hrs.	Time, hrs.	H_2S as mg./l.	Time to reach 700 mg., hrs.
None	8.36	43	10	>169	168	21	420	100	13	166
		100	52		262	29		167	743	
		169	190		475	1121				
1	8.17	100	196	126			190	76	27	129
		169	1118		168	542		100	21	
		262	1212		262	1232		167	1734	
		528	1255		528	1636		864	1673	
2	8.34	100	21	205	168	211	324	100	27	153
		169	121		262	517		167	944	
		262	1285		475	1090		261	1174	
4	8.39	100	13	>262	168	13	>475	100	12	>261
		169	12		262	18		167	14	
		262	20		475	18		261	22	

* Medium 220 was used as the basal medium.

Further results on the effect of the concentration of NaCl are given in Table IX. In this experiment the effect of NaCl on the rate of H_2S formation rather than on the total amount formed was determined. Medium 220 which has been found very favorable for the rapid production of high concentrations of H_2S was used. This medium contains 1 per cent NaCl and the effect of varying the NaCl content from no NaCl to 4 per cent NaCl was studied. Medium 220 contains 3.18 per cent of salts in addition to 2.1 per cent of sodium lactate. It is clear from the data that for all three strains (two marine and one non-marine) the medium containing 1 per cent of NaCl is definitely better than the others tested. Better growth was obtained with 2 per cent NaCl than with none, and no development occurred with 4 per cent during the period under which the cultures were kept under observation.

CONDITIONS GIVING MAXIMUM CONCENTRATIONS OF HYDROGEN SULFIDE

The composition of a medium, No. 220, which furnishes a proper environment for the rapid formation of over 2000 mg. per liter of H_2S has already been given under Materials and Methods. In the preceding section some of the experiments leading to the use of 1 per cent NaCl, rather than more or less, in this medium have been described. It is not feasible to discuss all the media variations that have been tested but some of the more important experiments contributing to the development of medium No. 220 will be discussed in this section. Data will also be given showing how the concentrations of H_2S produced by a given strain can be increased markedly by frequent transfers.

As stated in the introduction, when these experiments were started it was considered that, according to the work of Baars (1), marine forms were capable of producing much higher concentrations of H_2S than non-marine forms. The maximum reported for non-marine forms was only 794 mg. per l. as compared to 1448 for marine forms. In the present experiments it was found that bacteria of non-marine origin developed readily in media containing considerable quantities of NaCl and other salts. It was relatively easy to obtain concentrations higher than 794 mg. per liter for non-marine forms. Numerous examples of strains yielding concentrations above this value are included in the tables already given. C-13, C-13A, S2-11, S2-11A of Table VII, C-12, S2-10 of Table VIII, and CS-15A of Table IX all represent strains non-marine in origin. It was considerably more difficult to obtain concentrations higher than 1400 to 1500 mg. per liter.

EFFECT OF CALCIUM CARBONATE

The first culture medium yielding more than 1500 mg. per liter of H_2S was one to which $CaCO_3$ had been added and thus it had an initial pH value higher than had been used previously. Accordingly, the effect of the addition of $CaCO_3$ and of a higher initial pH on the total amounts of H_2S produced was studied rather extensively. Some of these data are given in Tables X, XI, and XII. The composition of medium 21, used in the data of Table X has been given in a footnote to Table IV. Medium 66 contains all the ingredients of 21 plus 3.5 g. sodium lactate. In the tests reported in Table X the media without $CaCO_3$ were adjusted so that they

TABLE X

EFFECT OF ADDING $CaCO_3$ TO THE MEDIUM ON THE TOTAL AMOUNT OF HYDROGEN SULFIDE PRODUCED BY SULFATE-REDUCING BACTERIA

Strain and transfer No.	Days	H_2S produced as mg. per liter			
		Medium 21		Medium 66	
		No $CaCO_3$ pH 6.96	$CaCO_3$ pH 7.63	No $CaCO_3$ pH 6.97	$CaCO_3$ pH 7.68
Z2-8	16	896	1399	1505	1654
31-4	15	876	1278	849	1326
NB-8A	15	564	1310	463	842
22-6	15	721	1145	879	1148
NB-7C	13	269	469	101	99
Z2-6	13	1309	1364	1449	1543

had a pH value of 6.96 and 6.97 respectively after autoclaving. The media to which 1.00 g. of $CaCO_3$ per liter had been added had pH values of 7.63 and 7.68 after autoclaving. It is clear from the data in this table that the addition of the $CaCO_3$ increased the amount of H_2S obtained since such an increase was noted in 11 of the 12 comparisons available.

Nutrients better than No. 21 were later found and the desirability of adding $CaCO_3$ to the improved nutrients was investigated. One such medium is No. 95 which has the same composition as No. 220 except that it contains 36.94 g. of Na_2SO_4 per liter instead of 18.25 g. and contains no $CaCO_3$. Experiments in which the total amount of H_2S formed in this medium was compared with the amounts formed in the same medium with 0.28 g. $CaCO_3$ (No. 101) and 1.12 g. $CaCO_3$ (No. 105) per liter added are given in Table XI. In every case but two the cultures in medium 95 gave values lower than in either medium 101 or 105. The superiority of

TABLE XI

FURTHER EXPERIMENTS ON THE EFFECT OF ADDING $CaCO_3$ ON THE TOTAL AMOUNTS OF HYDROGEN SULFIDE PRODUCED BY SULFATE-REDUCING BACTERIA

Strain and transfer No.	H_2S produced as mg. per liter			
	Days	Medium 95 No $CaCO_3$ pH 7.22	Medium 101 0.28 g. $CaCO_3$ pH 7.88	Medium 105 1.12 g. $CaCO_3$ pH 8.28
Z2-8A	12	1413	1511	1562
S2-8A	12	973	1312	925
Z3-9	13	976	1246	1230
C-12	18	1179	1278	1208
S2-10	17	1021	992	1102
1M-3	19	1326	1530	1544
24-5	18	1012	1111	1186
P11-4	19	1252	1476	1605
Z2-9A	14	985	—	1520
S2-9A	14	1036	—	1503

media 101 and 105 over 95 is highly significant as shown by ranking methods (8, 20). No difference between 101 and 105 is apparent. In this series S2 and C are strains non-marine in origin.

In still another experiment, the data of which are given in Table XII, the effect of medium 92, containing $CaCO_3$, was compared with the same medium adjusted to a pH comparable to that resulting from adding $CaCO_3$. The tests were run with ten strains and in every case medium 92, containing $CaCO_3$ gave a higher total quantity of H_2S.

TABLE XII

COMPARISONS BETWEEN TOTAL AMOUNT OF H_2S PRODUCED WITH AND WITHOUT $CaCO_3$ AT pH VALUES OVER 8.0

Strain and transfer No.	H_2S produced as mg. per liter		
	Days	Medium 92 $CaCO_3$ pH 8.08	Medium 141 No $CaCO_3$ pH 8.23
24-6B	11	1033	1012
C-15B	11	1175	1050
R2-3B	35	1738	1474
23-6	31	1663	1341
29-6	22	1715	1155
Z3-11C	20	1271	831
31-8	21	1602	1349
Z3-11D	30	1133	983
NA-12	29	898	813
NB-12	30	920	780

EFFECT OF CONTAMINANTS

As stated earlier for most of these tests each time transfers were made 1 cc. of the culture was also inoculated into medium 22 maintained under aerobic conditions. This medium supported good growth when inoculated with crude cultures. After a number of transfers from media in which 1000 or more milligrams of H_2S per liter were formed, the culture in medium 22 maintained under aerobic conditions remained clear, indicating the absence of contaminants able to develop under these conditions. The number of transfers necessary before this took place varied with different cultures presumably largely because of differences in the nature of accompanying bacteria and also differences in the amounts of H_2S produced in the cultures and variations in the length of time cultures were maintained with high concentrations of H_2S before transfer. After a given strain was free of the contaminating bacteria a culture in medium 22 was still carried along at each transfer to keep a constant check on the continued absence of contaminants. Occasionally after a number of passages during which no development occurred when cultured in medium 22 slight development could again be noted. This could have been the result of the introduction of a contaminant during transfer and possibly also the result of picking up bacteria around the neck of the bottles since the tops were not protected from the air during incubation and it was not practical to flame the tops when removing material for transfer since the bottles were full of liquid and also because hydrogen sulfide is inflammable. Thus it is possible that occasionally bacteria may have been picked up on transfer which would not grow in the media used for the sulfate reducers but could develop in the aerobic medium. In any event, if any contaminant was indicated at such a stage it was usually evident only by a very slight development in the aerobic medium.

Microscopic examination of a limited number of cultures free of bacteria developing when cultured aerobically in medium 22 has also indicated these cultures to be free of contaminants.

With crude cultures, representing original inoculations and the first few transfers thereafter before concentrations of H_2S of about 1000 mg. per liter or more were built up, it was apparent that the presence of contaminants reduced the amount of H_2S produced. However, after about 1200 to 1400 mg. per liter were produced in a series of cultures, contaminating bacteria if indicated to be still present by slight development in medium 22 did not seem to have any pronounced effect on the production of H_2S. Thus if a number of strains were being maintained simultaneously under conditions in which they gave about the same concentrations of H_2S, 1200 to 1400 mg. per liter, it was not possible to pick out the cultures that were going to be highest in H_2S content on the basis of whether or not slight development took place when cultured aerobically in medium 22.

In this connection the results with a large series of cultures in medium No. 92 are of interest. For a considerable period all strains were maintained as a routine in this medium. All H_2S values obtained in this medium were tabulated and separated into two groups, one in which medium 22 remained clear under aerobic conditions and one in which there was some development, however slight, under these conditions. Values obtained for cultures maintained for less than ten days or for cultures in which less than 100 mg. of H_2S were produced in ten days were eliminated. On this basis the average concentration of H_2S obtained with 293 individual cultures in which no growth occurred in an accompanying culture in medium 22 under aerobic conditions was 1257 mg. per liter. In the series in which some cloudiness occurred in the aerobic test an average value for 201 cultures of 1247 mg. of H_2S per liter was obtained when all cultures which had been transferred less than six times since the original inoculation were excluded. If 34 cultures which had been transferred only five times were included this average was reduced to 1198 mg. per liter.

It is of interest also to compare the two series in regard to the number of cultures in which high values were obtained. The 293 cultures in the group in which no development occurred in medium 22 included 50 cultures or 17.1 per cent in which values for H_2S of over 1500 mg. per liter were reached. The 201 cultures in which it was indicated that contaminating bacteria were present included 37 cultures, or 18.4 per cent in which the concentration of H_2S exceeded 1500 mg. per liter.

It thus appears clear that the failure to obtain consistently high values (over 1500 mg. per liter) in these studies is not the result of lack of proper purification of the cultures. It is natural to look for an explanation in a possible difference in H_2S tolerance among the various strains. Indeed, in these series certain strains were found to be included in the high yielding cultures in greater proportion than called for by the relative frequency with which they were included in the tests. However, since the purpose of the experiments was to reach maximum concentrations of H_2S, there was a tendency to favor high yielding cultures by more frequent transfer. Examination of the conditions associated with the formation of high concentrations of H_2S suggested that a relationship exists between high yields, transfers at shorter intervals (less than ten days rather than from ten days to three weeks or more), and therefore rate of H_2S formation. The amount of H_2S that would be produced in a given culture was not directly associated with the age of the culture from which it was transferred but rather with the frequency with which a number of the preceding cultures had been transferred. Relationships between the rates of H_2S formation and total concentration produced will be discussed in a succeeding section. First a study of the effect of sulfate concentration, which led to the abandonment of medium 92 for routine use, will be presented.

EFFECT OF SULFATE CONCENTRATION

Medium No. 92 contains 36.94 g. of Na_2SO_4 and 2.00 g. of $MgSO_4 \cdot 7H_2O$ per liter or sufficient to yield 9147 mg. of H_2S per liter although only enough lactate is present to permit the formation of 3192 mg. per liter if all the lactate is oxidized to acetate. Medium 92 was used for a considerable period of time without questioning the feasibility of the high sulfate content since earlier experiments had indicated the desirability of the presence of sulfate in excess and because higher values for H_2S were being obtained with this medium than with others previously tried. When experiments were carried out on the effect of sulfate concentration it became readily apparent that an improved medium would result if the amount of Na_2SO_4 present was decreased. The data are reported in Table XIII. Medium 220, containing 18.25 g. of Na_2SO_4, sufficient with the $MgSO_4$ also present, to permit the formation of 4655 mg. per liter, is markedly better than medium 92. There is also an indication that medium 222 may be somewhat better than 220 but the use of this medium was not adopted since it contains only sufficient sulfate to give 2467 mg. per liter of H_2S.

TABLE XIII

EFFECT OF VARYING THE AMOUNT OF Na_2SO_4 PRESENT ON THE RATE OF H_2S FORMATION

Medium			H_2S production								
			P11-10			18-12A			S2-19A		
No.	Na_2SO_4 content, g./l.	pH	Time, hrs.	H_2S as mg./l.	Time to reach 700 mg., hrs.	Time, hrs.	H_2S as mg./l.	Time to reach 600 mg., hrs.	Time, hrs.	H_2S as mg./l.	Time to reach 700 mg., hrs.
222	9.13	8.23	42	10	111	26	10	123	33	7	110
			66	14		44	31		68	28	
			118	818		68	170		120	935	
			188	1230		189	1225		189	1257	
220	18.25	8.39	42	10	130	26	14	139	33	12	118
			66	9		44	20		68	24	
			118	579		68	44		120	768	
			188	1172		189	1131		189	1222	
92	36.94	8.55	42	5	>188	26	10	157	33	10	168
			66	10		44	10		68	14	
			118	10		68	28		120	141	
			188	17		189	1060		189	988	
224	73.0	8.76	42	5	>34 da.	26	7	>139	33	7	>68
			66	12		44	7		68	7	
			34 da.	nt*		68	7				
			34 da.	nt		189	7				

* nt = Examined after 34 days for possible H_2S formation but not titrated.

After the results given in Table XIII became available, medium 220 was used as the routine control medium and the studies on the relationships of frequency of transfer, rate of H_2S formation, and total quantity produced suggested by the results obtained with medium No. 92 were carried out with the improved medium.

RELATIONSHIP BETWEEN RATE OF FORMATION OF H_2S AND TOTAL QUANTITY PRODUCED

The simplest and most unequivocal proof that high total concentrations of H_2S are associated with more frequent transfers and therefore more rapid rates of H_2S formation as suggested by an examination of the data obtained with a large series of cultures maintained in medium 92, can be obtained by showing that various strains which have as yet not produced these high concentrations can be induced to do so by proper manipulation. Such tests were carried out with all strains under cultivation in this laboratory (over 40) and in each case vigorous cultures producing over 2000 mg. of H_2S per liter were obtained.

The results with three strains are given in Table XIV. The first strain, labeled NA, was originally obtained from stagnant fresh water near Nyack, N. Y. After being transferred 18 to 19 times over a period of a year or so the maximum concentrations of H_2S that had been reached with the various media tested were around 1200 to 1400 mg. per liter. Cultures represented by transfer numbers 18A and 19 shown in the table yielded a maximum of 1319 mg. per liter after 29 to 35 days. Cultures of transfer number 20A showed loss of vigor, apparently the result of being transferred from a culture 35 days old. (Too much delay in transfer of cultures high in H_2S content sometimes seriously delayed subsequent growth.) Cultures in 20A developed only 593 mg. per liter after 22 days. On instituting a program of more frequent transfer vigor was increased rapidly. Thus 21A transferred from 20A at five days, although the H_2S content was only 91 mg. per liter at that time, produced 1449 mg. per liter in 15 days. Subsequently after a number of further transfers at intervals of two to ten days this strain was able to produce 1160 mg. in two days, 1687 to 1787 mg. in three days, and 2154 to 2588 mg. in four to thirteen days.

The other two strains, R3 and 50, covered in Table XIV, are of interest because of the high production of H_2S in the original crude cultures. Culture R3 was obtained from the Atlantic Ocean at Newport, R. I. In the absence of equipment to take samples in mud at some distance below the surface, the sample taken consisted of bits of seaweed, Irish moss, gathered at the shore level and placed into a sterile bottle with enough sea water to completely fill the bottle. After two days 1 cc. of water from this bottle was inoculated into each of two bottles containing medium 92. These two

TABLE XIV

DATA SHOWING THE INCREASE IN RATE OF H_2S FORMATION AND INCREASE IN THE MAXIMUM AMOUNTS PRODUCED BY THREE STRAINS AS THE RESULT OF FREQUENT TRANSFERS

NA

Transfer No.	H_2S production	
	Time, days	H_2S as mg./l.
18A	29	1216*
	29	1271
19	35	1319*
	35	1219
	35	1235
20A	5	91*
	22	593
21A	3	74
	10	1256*
	15	1449
22A	3	909
	4	1106*
	32	1573
23B	10	1136*
	16	1293
	28	1728
24B	4	1077
	5	1487*
	18	1929
25	3	1687*
	14	2347
26	3	1782
	4	2154*
	11	2292
27	2	1160*
	8	2460
	15	2494
28	3	1707
	6	2425
	13	2588

R3

Transfer No.	H_2S production	
	Time, days	H_2S as mg./l.
Original cultures	15	1046
	18	1217*
2A	11	1317*
	11	1328
3A	16	1386*
	31	866
4	15	1259*
	15	1239
5	24	1420*
	24	1400
6	3	44
	4	31
	5	193
	10	1107
	58	1356*
7	8	351*
8	4	440*
	13	1389
9	3	370*
	9	1327
10	4	577*
	18	1851
11	3	457
	7	2045

50

Transfer No.	H_2S production	
	Time, days	H_2S as mg./l.
Original culture	16	1332*
2	7	1335*
3	5	1244*
	8	1520
4	3	1132*
	8	1869
5	6	2120
	11	2167

* Culture transferred in each case.

cultures are the only ones covered in Table XIV in which medium 92 was used rather than 220. After 15 and 18 days the cultures had formed 1046 and 1217 mg. per liter of H_2S. On being transferred a number of times at intervals of 11 to 24 days the maximum concentration formed was only about 1400 mg. per liter. As in the NA series maintenance for a long period at relatively high concentrations of H_2S, namely for 58 days with a final concentration of 1356 mg. per liter, markedly reduced the concentration of H_2S that was produced on transfer. As in the case of the NA strain one transfer at a shorter time interval restored productivity to about 1400 mg. per liter. After several more transfers at three- to four-day intervals a culture which produced over 2000 mg. per liter in seven days was obtained.

Strain 50 was chosen for inclusion in Table XIV because of the small number of transfers required to obtain cultures producing over 2000 mg. per liter. Strain 50 represents reinoculation from core sample No. 30 originally received March 26, 1948. Inoculation into medium 220 with 1 to 2 g. of powdered material (stored in a sterilized glass stoppered bottle since its receipt) on April 25, 1949 gave a culture which reached a concentration of 1332 mg. per liter in 16 days. Only slight development occurred in the case of a simultaneous inoculation into medium 22 cultured aerobically. On the first transfer and other transfers thereafter the aerobic test showed apparent freedom from contamination and after relatively few transfers at intervals of three to seven days concentrations over 2000 mg. per liter were reached.

TABLE XV

DATA SHOWING RELATIONSHIP BETWEEN RATE OF H_2S FORMATION AND TOTAL CONCENTRATION PRODUCED

Culture and transfer No.	Maximum concn. of H_2S reached, mg./l.	Time to reach approx. 50%, hrs.
Z2-14D	2080	77
33-17A	2172	72
33-22A	2295	62
R4-12	2479	56
27-18	2351	57
33-29	2200	62
R4-18	2545	41
33-30	2619	47
S3-27	2286	46
R4-20A	2298	63
33-37	2213	64
33-39	2368	44
Av.		58
22-16A	1805	114
33-8	1806	83
Z2-19C	1924	76
Z2-20C	1750	132
33-13C	1956	59
R2-14	1844	104
33-17	1985	62
33-14E	1877	130
R4-26	1956	66
Av.		92

Culture and transfer No.	Maximum concn. of H_2S reached, mg./l.	Time to reach approx. 50%, hrs.
R2-10B	1551	128
R2-12	1619	192
PB22-16A	1606	99
18-16D	1642	58
Z2-26	1598	124
R4-22	1741	47
Z2-19B	1690	137
33-7	1579	100
18-11D	1627	131
Z2-22	1734	129
38-11	1636	190
R2-8C	1699	157
Av.		124

The relation between rate of H_2S formation and total quantities produced can also be illustrated by the data in Table XV. The data from all series in which time relation studies were carried out with medium 220 in which enough points were available to draw a curve and obtain a reasonably accurate estimate of the time required to reach about 50 per cent of the total concentration produced were gathered together and separated into groups representing varying final concentrations. Thus in the first part of Table XV are listed 12 series in which the final concentration reached was over 2000 mg. per liter. The time required to reach about 50 per cent of the concentrations involved averaged 58 hours and varied only from 41 to 77 hours. In the case of nine series in which the final concentrations ranged from 1750 to 1985 mg. per liter the time required to reach the 50 per cent point averaged 92 hours and with 12 series in which the final concentrations ranged from 1551 to 1741 mg. per liter the average time to reach the 50 per cent point was 124 hours. With 34 series, not listed in the table, giving values for the final concentrations of H_2S between 1126 and 1495 mg. per liter the average time required to reach the 50 per cent point was 123 hours.

Of significance is the fact that in no case in which over 2000 mg. per liter were formed was the rate of development slower than indicated by 77 hours for the 50 per cent point. In some cases fairly rapid development to the 50 per cent point was given by cultures not reaching such high values. This is understandable because rapid development takes place if cultures are transferred at the proper stage but production of over 2000 mg. per liter took place in these experiments only after a series of several such transfers.

The addition of peptone or yeast extract to cultures in medium No. 220 capable of producing 2000 to 2500 mg. per liter of H_2S in three to four days produced a marked stimulation of the rate of H_2S formation but not any increase in the total amount formed. These results will be covered in a succeeding paper. The addition of peptone or yeast extract to a medium used for the routine cultivation of sulfate reducers in glass stoppered bottles is not recommended because of the ease with which such cultures become contaminated.

SUMMARY

Sulfate-reducing bacteria (*Desulfovibrio desulfuricans* [Beij.] K. and v.N.) have been obtained from the inner portions of drill cores from depths of 700 to 900 feet in sulfur-bearing salt domes in Texas.

Experiments have been carried out with strains obtained from the above sources, from other sources representing a marine environment, and from a variety of non-marine sources to determine the highest concentrations of hydrogen sulfide that could be produced by this organism. It was

found that the previously reported maxima, 1468 mg. per liter for marine forms and 794 mg. per liter for non-marine forms, could be exceeded by both forms with the use of appropriate media. A medium was devised which appears equally suitable for both forms and which permits the development of concentrations of hydrogen sulfide of 2000 to 2500 mg. per liter. The improved medium contains the following salts expressed as grams per liter:

NH_4Cl	1.00 g.
$MgSO_4 \cdot 7H_2O$	2.00 g.
Na_2SO_4	18.25 g.
K_2HPO_4	0.50 g.
$CaCl_2 \cdot 2H_2O$	0.10 g.
Sodium lactate	21.0 g.
NaCl	10.0 g.
$CaCO_3$	1.00 g.
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Trace

For both marine and non-marine forms more rapid development occurred in 1 per cent of NaCl than with no NaCl or 2 or more per cent of NaCl. The presence of $CaCO_3$ is important and better results are obtained when $CaCO_3$ is used than when similar pH values are induced by the addition of NaOH. The use of larger amounts of Na_2SO_4 reduces the rate of H_2S formation without increasing the total concentration reached.

In order to obtain the formation of such high concentrations of H_2S it is necessary not only to use the proper medium but to have the bacteria in a vigorous condition. In these experiments it was necessary to pass cultures through a number of transfers at frequent intervals, preferably not more than three to five days apart, before productivity above 2000 mg. per liter was obtained. Thus, contrary to the conclusions of some previous workers, the production of high concentrations was associated with rapid rates of H_2S formation. All strains grown in this laboratory, over 40 in number, both non-marine and marine in origin, produced over 2000 mg. per liter when grown in suitable media and transferred at frequent intervals.

The high concentrations of H_2S produced in appropriate media tended to kill out contaminating organisms in enrichment cultures so that after five or six transfers from media in which over 1000 mg. per liter of H_2S had been produced facultative organisms developing in a medium maintained under aerobic conditions and containing glucose, peptone, and sodium lactate were no longer present. Microscopic examination of a limited number of such cultures also indicated the absence of contaminants.

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STIMULATION OF HYDROGEN SULFIDE PRODUCTION BY SULFATE-REDUCING BACTERIA

Lawrence P. Miller

Sulfate-reducing bacteria (*Desulfovibrio desulfuricans* [Beij.] K. and v.N.) will develop readily in liquid media to which no other organic compound except sodium lactate or some other suitable hydrogen donor has been added. It has been difficult, however, to obtain growth on solid media until Starkey (5) introduced a medium containing peptone and dextrose. Rittenberg (4) found that dextrose was not essential but that equally good results were obtained with peptone and sodium lactate. It would thus seem that, at least under these conditions, peptone favors the growth of the sulfate reducers. Rittenberg also tested peptone in liquid media as a hydrogen donor and as a hydrogen acceptor and obtained some development in both media but he does not report any observations on an effect of peptone on the *rate* of growth in the presence of sulfate and sodium lactate.

Bunker (1) found that yeast, yeast water, sterile filtered yeast culture in wort, and wort itself stimulated the growth of sulfate reducers. He also observed that the same strain kept in pure culture for several years was not stimulated consistently by these supplements. More recently Butlin, Adams, and Thomas (2), working in Bunker's laboratory, found that the addition of 0.1 per cent Difco yeast extract greatly stimulated growth and that with such a medium considerable turbidity is obtained within 48 hours. No quantitative data are given.

In a previous paper (3) from this Institute it was shown that with appropriate media and growing conditions sulfate reducers will produce 2000 to 2500 mg. of H_2S per liter in three to four days without stimulants. However, in experiments carried out concurrently with those reported and largely completed before the work of Butlin *et al.* (2) became available, it was found that adjuvants such as peptone, yeast extract, potato juice, milk, liver extract, and a number of other materials were powerful stimulants under all conditions tested. Under unfavorable conditions the effect of these adjuvants often appeared to be qualitative in that no growth took place in the controls. These experiments with adjuvants are reported in the present paper. A brief summary is also included of tests carried out with a total of about 35 amino acids, vitamins, and other growth factors in an attempt to identify the substance or substances in these adjuvants responsible for the stimulation.

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MATERIALS AND METHODS

Details as to the techniques used in growing the bacteria, transferring cultures, and determining quantitatively the hydrogen sulfide produced are reported in a previous paper (3). In all cases time studies were carried out, *i.e.*, several cultures were set up in each medium and individual cultures were titrated at different time intervals. The data were plotted to give a curve from which it was possible to read off the time required to yield about 50 per cent of the total amount of H_2S formed. Since the 50 per cent point occurs on the vertical portion of the typical bacterial growth curve it can be determined with reasonable accuracy and serves as a convenient means of comparing growth rates in various media.

In most of the experiments reported the basal medium was that described previously as No. 220. It contains the following ingredients in grams per liter: NH_4Cl 1.00, $MgSO_4 \cdot 7H_2O$ 2.00, Na_2SO_4 18.25, K_2HPO_4 0.50, $CaCl_2 \cdot 2H_2O$ 0.10, sodium lactate 21.0, NaCl 10.0, $CaCO_3$ 1.00, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ trace.

Strains of bacteria used in these studies included forms both marine and non-marine in origin. Those numbered from 18 to 27 and those in which the number is preceded by P or PB were obtained from drill cores from sulfur-producing salt domes in Texas. Strains numbered S1, S2, and S3 represent non-marine forms obtained from Dr. R. L. Starkey and those numbered Z2 and Z3 are marine forms furnished by Dr. Claude E. ZoBell. Those designated as C, CS, and 33 and 34 were derived from soil at the Institute, and strains labelled R2 and R4 were obtained from the ocean at Newport, R. I. Medium 220 was equally suitable for both marine and non-marine forms and under the proper conditions equivalent growth responses resulted.

EXPERIMENTAL RESULTS

Effect of peptone. A number of the tests carried out with peptone are listed in Table I. Experiments in which 9 to 10 grams of peptone per liter were used are shown in the first part of the table and the individual tests are arranged in order of decreasing rate of H_2S formation by the controls. Differences in the rate of growth of the controls were attributable, primarily, to variations in the age of the inoculum used. It is apparent that peptone was stimulatory in all cases. The greatest degree of stimulation was obtained from cultures of low vigor in the basal medium. These cultures developed high concentrations of H_2S almost as rapidly as more vigorous cultures by addition of peptone to the medium. The effects of lower concentrations of peptone are shown in the second part of the table and it is seen that measurable responses were obtained with concentrations less than 10 milligrams per liter.

The greatest stimulation from peptone was obtained when bacteria

TABLE I

EFFECT OF PEPTONE IN INCREASING THE RATE OF H_2S PRODUCTION BY SULFATE-REDUCING BACTERIA

Concn. of peptone, g./l.	Strain and transfer No.	Time to reach 50% point in H_2S production, hrs.		Concn. of peptone, g./l.	Strain and transfer No.	Time to reach 50% point in H_2S production, hrs.	
		With peptone	Without peptone			With peptone	Without peptone
10.0	R2-6A	51	210	5.0	33-14F	38	130
9.6	S3-13	40	184	"	33-14E	38	101
10.0	22-16	33	169				
10.0	22-16A	37	155	"	Z2-24D	40	77
9.6	34-5	32	152	"	33-17	41	62
10.0	31-15	31	151				
"	CS-11	49	151	1.0	22-17	69	116
"	P7-9	38	146	"	CS-15	70	113
"	Z2-19B	37	137	"	R4-26	57	66
"	S2-21A	37	136	"	33-37	42	64
"	18-10	47	132	0.100	Z2-20C	118	132
"	18-11C	30	124	"	C-24B	63	97
"	24-12	33	118	"	PB22-10	63	88
"	22-17	36	116				
"	CS-15	29	113	0.165	22-16A	65	114
"	S3-14A	29	104	0.075		98	
"	NB-17A	33	106				
"	33-7	32	100	0.165	18-14	57	109
"	P11-12	33	97	0.075		100	
"	Z3-13A	31	93				
9.6	S2-14	30	79	0.075	22-15D	79	134
10.0	S2-15A	30	77	0.0075		77	
"	33-48	26	52	0.00075		93	
"	33-49A	35	49				
"	R4-35	29	44	0.075	18-11D	81	131
				0.0075		90	
				0.00075		91	

were grown under unfavorable conditions. Thus in a study on the effect of temperature, no growth was obtained with two strains on peptone-free medium after 23 days at 20° C. and at an alternating temperature of 15° to 30° C. (8 hours at 30° followed by 16 hours at 15°; weekends at 15°), while corresponding cultures to which 9.6 grams of peptone per liter had been added produced 825 and 1235 mg. of H_2S per liter, and 1160 and 1211 mg. per liter respectively, in 12 days. Similarly in tests conducted with peptone-free media adjusted to pH 6.00, no development was noted after 34 days while 695 mg. of H_2S per liter were produced in 142 hours when peptone was present. In another experiment in which the effect of high concentrations of sodium sulfate in the media was studied concentrations of 73.0 g. per liter inhibited growth when no peptone was present while the time required to produce 700 mg. of H_2S per liter when peptone was added was 183, 157, and 198 hours for the three strains tested. In all of the above experiments, when growth occurred on peptone-free medium, H_2S production was stimulated by adding peptone.

Effect of yeast extract. Preliminary tests carried out with hot water extracts of dried yeast showed that stimulatory factors could also be obtained from yeast. Accordingly, comparisons were made between the effectiveness of peptone and yeast extract (both from Difco Laboratories) and it was found that yeast was somewhat more effective on a weight basis. Some of the results with yeast extract are summarized in Table II. Included are a number of tests at concentrations of 5.0, 1.0, 0.2, 0.1, and 0.05 grams per liter and also a number of experiments in which the effect of a wider range of concentrations was compared directly. A pronounced stimulatory effect is apparent. The data for the time necessary to reach the

TABLE II

STIMULATING EFFECT OF YEAST EXTRACT ON THE PRODUCTION OF H_2S BY SULFATE-REDUCING BACTERIA

Concn. of yeast extract, g./l.	Strain and transfer No.	Time to reach 50% point in H_2S production, hrs.	
		With yeast extract	Without yeast extract
5.0	33-14F	36	130
“	CS-15A	38	126
“	33-14E	34	101
“	Z2-14D	33	77
“	R4-20A	33	63
“	33-17	36	62
“	27-27	36	48
“	R4-22	28	47
“	33-30	35	47
“	S3-27	26	46
1.0	R2-12	61	192
“	24-13A	47	158
“	22-17	41	116
“	CS-15	38	113
“	R4-26	43	66
“		42	
“	33-37	36	64
“		37	
0.200	33-55	24	30
“	27-47	26	34
0.100	S1-22	109	139
“	Z2-20C	83	132
“	S3-17A	55	115
“	C-24B	58	97
“	PB22-10	49	88
“	33-53A	45	56
“	27-46	32	36
“	33-54	31	35
0.050	24-15	91	150
“	PB22-10A	80	131
“	P3-11	72	131
“	R2-10B	81	128
16.4	33-51	22	37
4.1		24	
1.0		25	
0.256		27	
0.064		30	
0.016		32	
0.004		35	
0.001		37	
16.4	27-44	25	39
4.1		24	
1.0		26	
0.256		30	
0.064		32	
0.016		34	
0.004		36	
0.001		37	
10.0	18-11C	30	124
5.0		30	
1.0		34	
10.0	P11-12	33	97
5.0		34	
1.0		38	
10.0	33-7	31	100
5.0		34	
1 0		36	

50 per cent point in the quantity of H_2S produced for two series in which a range of concentrations from 1 milligram to 16.4 grams per liter was tested, indicate an increasing effect from around 4 milligrams to over 4 grams per liter. Four-fold differences in concentration often decreased the time required to reach the 50 per cent point by only an hour or two. That these are real differences, however, is brought out by an examination of the data in Table III in which the actual titration values obtained at two time intervals are given. Higher values for each increase in concentration are evident for both time intervals at all concentrations except for the few lower members in the series and also with the exception that at the 43- to 44-hour period there was an indication that 16.4 g. per liter may have been less favorable than a somewhat lower concentration. It would appear from an examination of the data in Table III and from other data available that for the detection of slight effects it is advantageous to make comparisons when the cultures have produced from about 100 to 500 milligrams of H_2S per liter.

TABLE III

EFFECT OF CONCENTRATION OF YEAST EXTRACT IN NUTRIENT MEDIUM ON H_2S PRODUCED AT VARIOUS TIME INTERVALS

Concn. of yeast extract, g./l.	Strain and transfer No.	H_2S in mg./l. at two time intervals in hrs.		Strain and transfer No.	H_2S in mg./l. at two time intervals in hrs.	
		23–25	43–44		24–25	44
16.4	27-44	949	1998	33-51	1138	1938
4.1		743	2060		999	2216
1.0		659	1854		790	1988
0.256		517	1535		490	1787
0.064		227	1514		345	1595
0.016		167	1409		215	1469
0.004		122	1265		166	1396
0.001		114	1248		185	1290
0		170	1167		150	1313
10.0	P11-12	323	1274	33-7	344	1329
5.0		212	1218		226	1303
1.0		102	1021		107	1113
0		15	33		15	19

Effect of miscellaneous adjuvants. Results obtained in the examination of other materials as possible sources of the stimulating factor or factors present in peptone and yeast extract are shown in Table IV. It would seem that these factors are widely distributed since positive results were obtained with potato juice, milk, tomato plant juice, liver extract, yeast extract, autolyzed yeast, yeast hydrolysate, soy protein hydrolysate, and casein hydrolysate. For comparative purposes results with yeast extract and peptone used in the same series are included in the data presented. In view of the nature of the response curves one should be rather cautious in making comparisons on a concentration basis unless careful studies have been carried out but it seems safe to conclude that the data suggest high activity for potato juice and liver extract and for some of the protein hydrolysates.

Tests with chemically defined growth factors. The nature of the materials found to be stimulative suggests that the factor or factors responsible may be found among the amino acids or B vitamins. Extensive experiments have been carried out involving about 35 compounds which it was thought might possibly be associated with the stimulative effect. These have included the available amino acids, the vitamins belonging to the B group, and a number of other chemicals which have been shown to be necessary for the development of various bacteria. In no case has a stimulatory

TABLE IV

EFFECT OF SOME MISCELLANEOUS ADJUVANTS ON THE RATE OF H_2S FORMATION BY SULFATE-REDUCING BACTERIA

Adjuvant	Amt. added per l.	Strain and transfer No.	Time to reach 50% point in H_2S production, hrs.		Strain and transfer No.	Time to reach 50% point in H_2S production, hrs.	
			With adjuvant	Without adjuvant		With adjuvant	Without adjuvant
Peptone	1 g.	33-37	42	64	R4-26	57	66
Yeast extract			36			43	
Autolyzed yeast			54			52	
Brewer's yeast			48			49	
Yeast hydrolysate			36			38	
Soy protein hydrolysate			40			46	
Casein hydrolysate			37			46	
Yeast extract	5 g.	27-27	36	48	R4-22	28	47
Potato juice	100 cc.		40			32	
(peel)	10 cc.		40			42	
Potato juice	100 cc.		34			24	
(inside of tuber)	10 cc.		39			48	
Yeast extract	5 g.	R4-20A	33	63	S3-27	26	46
Milk	100 cc.		36			28	
Potato juice	100 cc.		35			27	
Mixture of all three	5 g., 100 cc. and 100 cc.		28			23	
Peptone	10 g.	Z2-19B	37	137	S2-21A	37	136
Milk	30 cc.		69			64	
Peptone	9.6 g.	S3-13	40	184	34-5	32	152
Tomato juice (leaves and stems)	50 cc.		97			89	
Potato juice	50 cc.		41			37	
Liver extract	7.5 cc.	R4-44	37	79	33-59	32	71
	0.38 cc.	33-58A	83	93	27-50A	37	49

effect equivalent to that of yeast extract or peptone been obtained, although a certain amount of stimulation has been noted in a few instances. From the results so far obtained it appears that a combination of several factors is probably involved. Further experiments are in progress.

DISCUSSION

Since the effect of these adjuvants is only stimulatory to the rate of H_2S formation and does not involve an increase in the total quantity of H_2S produced the magnitude of any differences observed between cultures with and without a particular adjuvant is dependent upon the time at which the comparison is made. It is apparent from the data presented that the relation between the concentration of adjuvant and the effect on the rate of H_2S production is not linear. Four-fold increases in concentration may only produce a decrease of an hour or two in the time required to reach the 50 per cent point in the total amount of H_2S formed. Preliminary attempts to plot the data in such a way as to result in a straight line relationship between concentration and degree of response indicate that for a rather limited concentration range and when taken at the proper time intervals a linear relation may be found between the quantity of H_2S formed and the logarithm of the concentration of adjuvant. Should it become desirable to use the stimulatory effect of these adjuvants in connection with an assay procedure either for known factors, the identity of which are still to be determined, or to follow progress in attempted isolation procedures, the possibilities of such a linear relationship should be further investigated.

Since the sulfate-reducing bacteria can produce high concentrations of hydrogen sulfide fairly rapidly (3) without the use of adjuvants, it would seem that the adjuvants merely supply a factor or factors which are formed slowly enough to prove limiting under the conditions used. It is reasonable to expect that the stimulation may involve more than one factor, since supplying one factor which may be limiting may result in turn in producing conditions in which a second necessary material may not be formed rapidly enough for optimum growth.

The magnitude of the effect of the adjuvants tested varied from an apparent qualitative response in which no growth took place in the controls to a relatively minor effect under the most favorable conditions in which the time required to reach the 50 per cent point in the total amount of H_2S produced was decreased only by about 10 to 15 hours or less. Butlin *et al.* (2) report that when 0.1 per cent of Difco yeast extract was added considerable turbidity was observed within 48 hours. In the tests with improved media and techniques in regard to optimum transfer-time intervals carried out at the Institute (3), the production of 1000 milligrams of H_2S per liter or more was obtained within 48 hours without the use of adjuvants. The possibility is thus suggested that further improvements in the nutrient medium may be achieved and that under such conditions adjuvants might not produce any further stimulation.

In the tests reported in the present paper on the effect of adjuvants, transfers into media containing adjuvants were always made from media free of such stimulants. It is obvious that transfers from media containing adjuvants might influence growth in subsequent media especially in view of the fact that measurable responses to adjuvants could be demonstrated at very low concentrations. Preliminary results indicate that when transfers are made from media containing 5 grams of yeast extract per liter into media containing the same amount of yeast extract, concentrations of H_2S of 1000 milligrams per liter can be reached in 24 hours.

SUMMARY

Peptone, yeast extract, liver extract, potato juice, milk, and a number of protein hydrolysates are powerful stimulants for the growth of sulfate-reducing bacteria (*Desulfovibrio desulfuricans* [Beij.] K. and v.N.) as measured by the rate of hydrogen sulfide formation.

The degree of stimulation obtained depended upon the growth rate of the controls and varied from an apparent qualitative effect, in which no growth occurred in the controls, to a decrease of about 10 hours or less in the time necessary to reach the 50 per cent point in the amount of H_2S produced under optimum conditions.

Under unfavorable conditions the effect appeared to be qualitative and the presence of adjuvants resulted in an extension of the temperature range and the pH range, and in an increase in the concentration of salts tolerated.

Maximum responses were obtained with peptone and yeast extract at concentrations of 5 to 10 grams per liter but measurable effects were observed at concentrations considerably less than 10 milligrams per liter.

Preliminary experiments conducted in an attempt to replace the adjuvants with known chemicals have as yet been inconclusive. The results suggest that more than one factor may be involved.

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